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Title:
Regulation of glutamate transport and inflammation in newborn brain injuries

Regulation of Glutamate Transport and Inflammation in Newborn Brain Injuries

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Abstract

Background:

Newborn brain injury is the leading cause of childhood neurodisability, including cerebral palsy (CP). Cystic periventricular leukomalacia (cPVL) selectively affects preterm newborns. Hypoxic-ischemic encephalopathy (HIE) affects term newborns with childbirth complications disrupting blood/oxygen supply to the brain. Glutamate excitotoxicity and inflammation are key mechanisms of injury. The role of genetic and epigenetic factors in regulation of glutamate transport and inflammation is unclear.

Hypotheses:

1) Preterm survivors with risk variants at the glutamate transporter (*EAAT2*) and key pro-inflammatory cytokines (*TNFα*, *IL1ß*, *IL6*) have higher risk of impairment vs those with either variant or none
2) Hypoxia-ischaemia (HI) alters transcription of these genes in the term-equivalent rat brain
3) This is mediated by a DNA methylation change in the brain, which correlates with methylation in blood

Methods:

In the APIP cohort (n=308), genotypes were tested for association with CP at 2y; secondary outcomes included cPVL, and standardised motor and cognitive assessments at 2y (Griffiths Scales) and 5y (Movement ABC; British Ability Scales, BAS).

In the rat study (n=42), the effects of HI on transcription of these genes were assessed in the cortex and hippocampus (qPCR), alongside DNA methylation of *EAAT2* and *TNFα* in cortex and blood (bisulfite pyrosequencing)

Results:

Cytokine variants are associated with cPVL and CP (*TNFα* -308) and the BAS cognitive score (*IL1ß* -511). Evidence was weaker for white matter injury (*IL6* -174), the non-verbal and verbal BAS subscales (*IL1ß* -511 and *EAAT2* -200/-181 respectively).

HI induced cytokine transcription in the rat brain, accompanied by astrogliosis and myelin injury. Evidence of suppression of glutamate transport in the cortex was very weak and not associated with DNA methylation changes.

Conclusions:

These exploratory studies support a role for neuroinflammation in neurological and neurodevelopmental impairment. Genetic and epigenetic biomarkers may facilitate early identification of high-risk newborns maximising chances of prevention and treatment.

Lay Summary

Background

Early life stresses such as prematurity, infections and complicated childbirths can damage the delicate developing brain. Newborn brain injury is the leading cause of childhood disabilities affecting movement (e.g. cerebral palsy), perception (e.g. blindness, deafness) and cognition (e.g. learning, language and memory). A better understanding of how brain injuries occur is needed to develop strategies to protect the brain. Two mechanisms are emerging as central: brain inflammation and disruption of the neurotransmitter glutamate, which enables communication between neurons. Neonatologists often observe that some babies cope worse than others upon similar stresses, raising the question of whether genetic differences might be important. For example, genetic make-up could affect the strength of inflammatory responses or the extent of glutamate transport disruption. Another unanswered question regards the actual mechanisms through which early life stresses trigger changes in the brain's responses, e.g. after a severe interruption of blood and oxygen flow (hypoxia-ischaemia) seen in complicated childbirths. DNA methylation is an interesting candidate mechanism, acting like a signature or molecular memory left on the DNA by stressful events. This signature can turn the dial up or down on the brain's responses and potentially trigger stronger inflammation or greater disruption of glutamate transport. Since it is not possible to carry out such investigations in babies, animal models become valuable.

Questions:

1) Do genetic variants affecting inflammation and glutamate transport influence the risk of brain injury and disability in a cohort of newborns born very prematurely in Bristol?
2) Does hypoxia-ischaemia alter inflammation and glutamate transport in the rat brain? Is this mediated by DNA methylation changes? Can DNA methylation changes reflecting the early stages of brain injury be picked up in blood (which is accessible in newborns)?

Results:

1) Genetic variants affecting the strength of inflammation are associated with risk of severe brain injury at birth, cerebral palsy at 2y and poorer cognitive scores at 5y. Evidence was more uncertain for genetic variants affecting glutamate transport in relation to verbal skills at 5y.
2) Hypoxia-ischaemia caused inflammation in the rat brain, at the beginning of the injury process. DNA methylation related to inflammation could not be assessed because of COVID-19-related disruptions and remains a priority for future work. Evidence was more uncertain for disruption of glutamate transport, and DNA methylation was not affected in brain or blood.

Conclusion:

The study supports a key role for inflammation in newborn brain injuries and related disability, as well as a contribution of genetic factors to risk, and encourages further research in this direction. Having this type of information ahead of time might help the family and healthcare professionals produce a personalised plan of care, which makes the most of the available prevention strategies and treatments to protect the brain (e.g. more frequent monitoring during pregnancy, labour, and childbirth; plan childbirth in a specialised hospital to avoid last minute transfers).

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: Silvia Pregnolato DATE: 16/12/2020

Plagiarism Statement

Excerpts from chapters 1 and 5 have been published in a review article co-authored by Prof Karen Luyt, Dr Ela Chakkarapani, Prof Anthony Isles (see Appendix). The first draft of the manuscript was written by me. Prof Karen Luyt and I contributed to the conception and design of the review. All authors revised, read, and approved the submitted version of the manuscript.

SIGNED: Silvia Pregnolato DATE: 16/12/2020

Karen Luyt

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Abbreviations

ADHD	Attention deficit hyperactivity disorder
aEEG	Amplitude-integrated electroencephalography
APIP	Avon Premature Infant Project
ASD	Autism spectrum disorders
BAS	British Ability Scales
bp	Base pair
bcDNA	Bisulfite converted DNA
BGT	Basal ganglia thalamus
cDNA	Complementary DNA
cffDNA	Cell free fetal DNA
CNS	Central nervous system
CNV	Copy number variant
CP	Cerebral palsy
cPVL	Cystic periventricular leukomalacia
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DAMPs	Danger associated molecular patterns
dNTP	Deoxyribonucleotide triphosphate
eQTL	Expression quantitative trait locus
EWAS	Epigenome-wide association study
FACS	Fluorescent-activated cell sorting
FDR	False discovery rate
FIRS	Fetal inflammatory response syndrome
GMFCS	Gross Motor Function Classification System
GWAS	Genome-wide association study
HI	Hypoxia-ischaemia
HIE	Hypoxic-ischemic encephalopathy
HWE/HWD	Hardy-Weinberg equilibrium/disequilibrium
IVH	Intraventricular haemorrhage
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
kb	Kilobases
M-ABC	Movement ABC Score
MACS	Magnetic-activated cell sorting
MAF	Minor allele frequency
mQTL	Methylation quantitative trait locus
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRS	Magnetic resonance spectroscopy
NEC	Necrotising enterocolitis
NICU	Neonatal intensive care unit
NTC	Non-template control
P7	Postnatal day 7
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RCT	Randomised controlled trials
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RT	Room temperature
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
TSS	Transcription start site
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
WS	Watershed

1 Background

1.1 Historical and global perspective on the care of the vulnerable newborn

Perinatal medicine has undergone remarkable advances in the last 70 years and has improved survival and quality of life of many vulnerable newborns, making neonatology the most rapidly developing specialty in paediatrics (1). While protection of the newborn brain is at the very core of these improvements, perinatal brain injuries are still leading causes of mortality and morbidity. This project focuses on the two of the most vulnerable populations of newborns. The first is represented by term newborns suffering from complications during labour and/or childbirth causing interruption in the supply of blood and oxygen to the brain leading to hypoxic-ischemic encephalopathy (HIE). The definition of HIE adopted in this project adheres to the current international consensus, which requires integration of clinical, biochemical, electrophysiological and neuroimaging data evidencing the intrapartum timing of the acute hypoxic-ischemic event leading to the encephalopathy (2, 3). The second population is represented by preterm newborns, who are often born with a variety of comorbidities, challenging an already vulnerable immature body and brain. The impact of prematurity on the brain involves a spectrum of abnormalities of the white and grey matter, collectively known as encephalopathies of prematurity. At the most severe end of the spectrum is cystic periventricular leukomalacia (cPVL), a severe form of preterm white matter injury characterised by necrotic cysts in the periventricular white matter large enough (≥2 mm) to be observed by cranial ultrasound at birth (4), and for which no postnatal treatment is currently available.

The care of the vulnerable newborn changed drastically from the 1950s and 1960s with the introduction of modern state-of-the-art neonatal intensive care units (NICUs) (5). Scientific and technological advances allowed substantial improvements in thermoregulation and supporting breathing and, as a consequence, sick newborns could be saved in intensive care rather than letting them die. Strict hygienic rules for staff and parents in handling the sick newborn, especially the most premature, were introduced to prevent spread of infections (6). In the 70s, collaborations between pioneering neonatologists and developmental psychologists led to the first preterm developmental programmes, focusing on optimising the postnatal environment to mimic the intrauterine environment, prevent the iatrogenic sequelae of the NICU, and ultimately improve wellbeing and development of these newborns (7-9).

This approach was further developed in the 80s, with implementation of the Newborn Individualized Developmental Care and Assessment Program (NIDCAP), which promoted an understanding of the importance of family involvement, mother-infant bonding, and kangaroo care, as well as a shift towards individualisation of care for each newborn based on detailed observation of behavioural cues during care (10, 11). By the late 1990s, technological advances in neonatal intensive care facilitated survival of neonates born as early as 23 weeks gestational age or as small as 500 g. This coincided with increased rates of preterm birth globally, possibly due to factors including increased maternal age, pregnancies complicated with maternal diabetes and hypertension, multiple pregnancies due to *in vitro* fertilisation and changes in obstetric practices (e.g. increase in c-sections) (as well as improved data collection) (12). While most preterm survivors grow into healthy children and adults, the preterm population as a whole is at risk for a variety of short- and long-term complications including brain injuries and impaired development, respiratory distress syndrome, retinopathy of prematurity and infection/inflammation (see 1.2). Preterm births represent a global health priority, not only because of the rising rates but also because of the lack of preventative treatments and effective screening strategies. Although there are general maternal health strategies (e.g. prenatal care, smoking cessation, pregnancy spacing, nutritional supplements during pregnancy) and some interventions in women with preterm labour (e.g. progesterone, cervical cerclage, antibiotics, tocolytics to slow contractions) that can reduce risk of preterm birth, the majority occurs unexpectedly in apparently low-risk women (13, 14). Nowadays, amongst preterm births, 60-70% are near term (34-36 weeks), 20% are moderately preterm (32-33 weeks), 10-15% are very preterm (28-31 weeks) and 5% are extremely preterm (<28 weeks) (15, 16). About 30% preterm births are medically induced (e.g. induction of labour or caesarean section) due to maternal or fetal indications (e.g. infection, pre-eclampsia); 45% preterm births occur due to spontaneous preterm labour, with onset of contractions preceding rupture of the fetal membranes; finally, 25% preterm births occur due to premature rupture of the membranes (PPROM) preceding onset of contractions (1, 15).

Despite the increase in rates of preterm birth, mortality of preterm newborns has decreased steeply in the last 70 years thanks to advances in perinatal care (17-22). This is most obvious when considering survival at the earliest gestational ages. In England, the EPICure studies showed that survival to discharge of extreme preterm newborns (22-25 weeks) improved from 40% in 1995 to 53% in 2006 (23, 24). In 2016, 8% of the newborns born alive at 22 weeks survived to 1 year in the UK; this reached 34% for those born at 23 weeks, 59% at 24

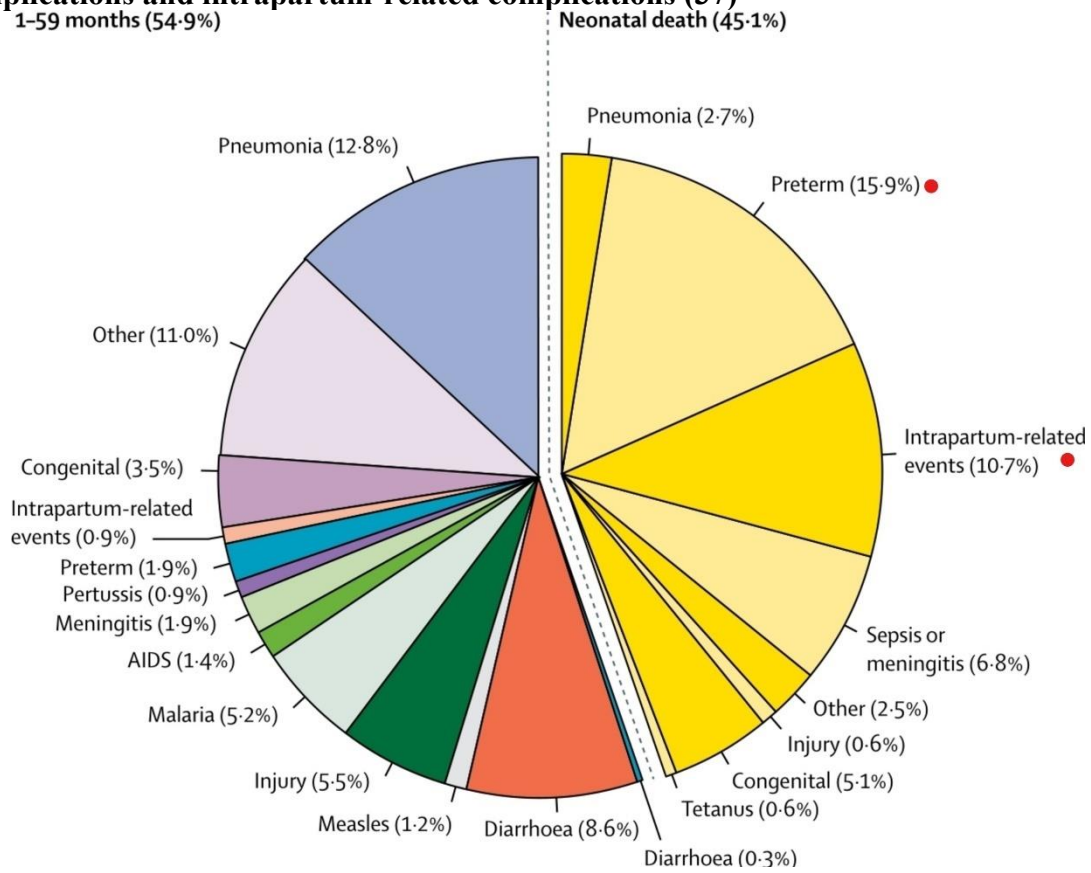
weeks, 74% at 25 weeks and 82% at 26 weeks (25). When the proportions are calculated based on the numbers of admissions to NICU rather than live births, they reach 54% for the newborns born at 22 weeks, 45% at 23 weeks, 63% at 24 weeks, 77% at 25 weeks and 84% at 26 weeks, highlighting the impact of intensive care for survival at the lowest gestational ages. Importantly, differences in active perinatal management exist from country to country, with lower survival (<1% at 22-23 weeks) in France, where the general policy is to provide palliative but not intensive care before 24 weeks (22). Nonetheless, the general trend for improved survival of preterm newborns has been observed not only in England, but also in France (EPIPAGE studies) (22, 26), Sweden (27, 28), the US (Neonatal Research Network studies) (29), Canada (30) and Japan (31), with relatively stable survival rates in Australia (32). The majority of newborns born very preterm (between 28 and 32 weeks, i.e. before 7 months of gestation) survive preterm birth, and, altogether, represent 85% of surviving preterm infants (33).

The more advanced type of data that could be obtained in the state-of-the-art NICU also allowed progress in the care of the term newborn suffering from birth-related complications. Until the 90s, a diagnosis of birth asphyxia had been based on the symptoms (e.g. breathing difficulties, bradycardia, low Apgar score, manifestation of the neurological syndrome) and the assumption that this was due to an interruption of oxygen and blood flow to the brain (hypoxia-ischaemia, HI) during labour and childbirth (intrapartum). It became increasingly clear that that these were non-specific signs of fetal compromise and that the proportion of cases with a true intrapartum HI insult preceding encephalopathy was much smaller (34). A diagnosis of HIE was introduced to describe these cases, with the requirement of objective clinical, biochemical and neuroimaging evidence of the intrapartum HI insult (2, 3). A generic diagnosis of neonatal encephalopathy was recommended for the remaining cases where causation was more ambiguous, including perhaps multiple hits of milder and intermittent or chronic hypoxia-ischaemia across the perinatal period, and/or a variety of infectious, genetic, metabolic or traumatic triggers (35).

Reflecting advances in perinatal care, the global neonatal mortality rate has halved from 5 million in 1990 to 2.5 million (7,000 neonatal deaths/day) in 2018 (36). Nonetheless, preterm birth complications and intrapartum-related complications remain the two leading causes of neonatal mortality globally, accounting for 35% (900,000) and 24% (600,000) neonatal deaths respectively (Figure 1.1). Next in line are sepsis (15%) and congenital abnormalities (11%) (36, 37). Amongst the 140 million newborns born alive every year, 15 million are born

preterm (<37 weeks of gestation), corresponding to 1 in 10 live births (12, 38). Of the 10 million newborns requiring some form of assistance to breathe in 2010, 1.15 million developed neonatal encephalopathy (8.5/1,000 live births), of which at least half died and at least a quarter developed some form of neurodevelopmental impairment (39). Asia and Sub-Saharan Africa account for the majority of neonatal deaths (90%), preterm births (80%) and neonatal encephalopathies (93%) (36, 38, 40). In high-income settings including Europe and North America neonatal mortality rates are much lower (1.5%) due to availability of advanced perinatal care. In the UK, intrapartum-related deaths have halved since the 90s due to such advances, and currently account for 5% neonatal deaths (41). In England and Wales, neonatal mortality has been in decline since records began in the 80s reflecting healthcare improvements, although a plateau was reached between 2017 and 2018 (2.8 deaths/1,000 live births). Preterm birth complications remain the leading cause of child mortality in England and Wales, accounting for nearly 50% neonatal deaths (42).

Figure 1.1 Global causes of under-5 deaths in 2015: focus on preterm birth complications and intrapartum-related complications (37)



Despite the trend for reduction in absolute numbers of neonatal deaths, the decline in neonatal mortality in high income-settings has been slower than that of older children (1-59

months) and the proportion of neonatal deaths amongst child deaths has actually increased from 40% in 1990 to 47% in 2018 (36). This has been an important obstacle in failing to meet the United Nations Millennium Development Goal to reduce childhood mortality by two-thirds in 2015 (43). In the UK, progress in reducing neonatal mortality has been slower than in comparably wealthy countries (EU15+: 15 Western Europe EU members in 2004, Norway, Canada and Australia) since the 00s, and specific UK Child Survival Goals have been set to address to improve UK survival by 2030 (44). Since the proportion of child mortality caused by neonatal deaths is increasing globally, the UN emphasise that "focusing on newborns is critical to further accelerating progress in child survival" (43). Moreover, since about a third of neonatal deaths occur in the first day of life and nearly three quarters in the first week (45, 46), "focusing on the critical periods before and immediately following birth is essential to saving more newborn lives" (36).

Survival of vulnerable newborns is highly dependent on place of birth and availability/practice of active perinatal management (47). Integration of multiple strategies will be required for progress, ranging from implementation of simple and cost-effective interventions, such as kangaroo care, support for breastfeeding and basic care for infections and breathing difficulties (12), to increased availability of more comprehensive obstetric and neonatal intensive care and specialised equipment (39, 48-50). In settings where advanced perinatal care is available, precision medicine may provide an opportunity for further improvement (51). Integrating molecular data (e.g. genomic, epigenomic, transcriptomic and proteomic data) alongside clinical and neuroimaging data has the potential to maximise tailoring of care to each newborn, extend the window for active management, improve the chances of prevention, and enable identification of novel neuroprotective approaches.

1.2 Short-term morbidities in the vulnerable newborn: perinatal brain injuries

1.2.1 The burden of morbidities: focus on perinatal brain injuries

A premature newborn is deprived of a crucial period of intrauterine growth and is born with a body that is immature in all its components. The consequences of these immaturity are particularly severe for organs that undergo crucial periods of growth in the third trimester of pregnancy, including the brain, lung, gut, and eye. Accordingly, common morbidities affecting the preterm newborn include respiratory distress syndrome, neonatal sepsis, necrotising enterocolitis, seizures, vision and hearing loss, and haemorrhagic and non-

haemorrhagic brain injuries (17, 38, 52-60). Term newborns tend to be less susceptible to the widespread multi-organ consequences and comorbidities of excessive immaturity, but more vulnerable to complications during labour and childbirth due to higher gestational length and birth weight. Indeed, an acute HI insult during a problematic labour and childbirth can have devastating and life-long consequences on the brain, especially in newborns exposed to other perinatal risk factors, such as infections or intrauterine growth restriction (61). The huge impact of preterm birth complications and intrapartum-related complications on morbidity is clear from WHO global estimates, which place these conditions in fourth and ninth place as leading causes of years of healthy life lost due to disability (i.e. over 102,000 and 67,000 disability-adjusted life years respectively), above causes such as congenital anomalies, HIV and tuberculosis (62). The societal burden is also economic, with an estimated £2.9 billion cost of preterm birth complications to the public sector in England and Wales in 2006 (63).

The human brain develops substantially after birth, especially in the first months and years of life, with the intracortical fibres of the cortex completing their myelination well into the third decade (64). Hence, all newborns are born with a brain that is inherently immature and vulnerable. While there are other causes of neurodevelopmental impairment (e.g. congenital abnormalities, e.g. chromosomal and genetic abnormalities or defects due to exposure to neurotoxins, stroke), as well as a range of risk factors (e.g. socioeconomic status, nutrition, cognitive stimulation, polygenic risk), perinatal brain injuries remain the leading cause of childhood neurodisability (65-67).

In 2015, a Brain Injuries Expert Working Group was formed and agreed on pragmatic definitions and classifications of perinatal brain injuries to be set as a standard in the UK (68). The aim was to facilitate systematic data collection for monitoring of trends in perinatal brain injuries and effectiveness of neuroprotective strategies. The consensus definition excluded congenital abnormalities and included all brain injuries detected during the neonatal unit stay, i.e. seizures, intracranial haemorrhage, perinatal stroke, hypoxic-ischemic encephalopathy, central nervous system (CNS) infections, bilirubin encephalopathy and periventricular leukomalacia. Since clear evidence of injury often emerges as the child grows older, it was acknowledged that an accurate measure of brain injuries is based on integration of short-term (neonatal) and long-term (childhood) outcomes. Using routinely collected NHS data, the group reported that the current rate of perinatal brain injuries in England, Wales and Scotland is 5 brain injuries/1,000 live births and that this rate has remained relatively stable

between 2010 and 2015. The national ambition announced by the UK Secretary of State for Health to halve this by 2030 places particular urgency on neuroprotection research (69, 70).

The vulnerability of preterm newborns is highlighted by the high incidence of brain injuries in this group (26 brain injuries/1,000 preterm live births) compared to term-born newborns (3.5 brain injuries/1,000 term live births) (68). On the other hand, term births represent the majority of births, making HIE the most common type of brain injury, with a rate of 2.6/1,000 live births in England. Next in line are seizures (1.9/1,000 live births), intracranial haemorrhage (1.1/1,000 live births), CNS infections (e.g. meningitis) (0.70/1,000 live births), bilirubin encephalopathy (0.60 /1,000 live births), perinatal stroke (0.14/1,000 live births) and finally cystic periventricular leukomalacia (cPVL) (0.3/1,000 live births). While most of these brain injuries affect both preterm and term newborns, over 80% HIE occurs in term newborns, whereas intracranial haemorrhage and cPVL disproportionally affect preterm newborns. Nearly all of the over 180 newborns who developed with cPVL in 2015 were born ≤ 33 weeks, and the rate of cPVL in this group reaches 12.4/1,000 live births. This is slightly lower than cPVL rate of 1.8% (18/1,000 live births) reported in preterm newborns born <34 weeks by the French EPIPAGE-2 study in 2011 (22). In clinical practice, a considerable degree of overlap is observed and newborns are often diagnosed with multiple types of brain injury (68), as for severe intraventricular haemorrhage (IVH) and cPVL (71-75).

1.2.2 Neuroimaging and brain injury patterns

Progress in neuroimaging techniques, with the introduction of MRI techniques alongside cranial ultrasound, has been key in understanding the structural correlates of childhood neurodevelopmental outcomes in the newborn period (76, 77).

1.2.2.1.1 Hypoxic-ischemic encephalopathy in the term newborn

Two main patterns of injury are associated with moderate to severe HIE (78). The first pattern (25%-75% cases) involves injury to the deep grey matter, including the basal ganglia and thalamus ("BGT pattern"), hippocampus and somatosensory cortex (which receives output from the motor cortex and integrates external sensory signals before movement initiation) (79-81). Injury may also involve the white matter, anterior and posterior limbs of internal capsule (ALIC and PLIC), brain stem and cerebellum, and extends further into the cortex in the most severe cases (79, 82-88). The BGT are regions of high metabolic demand and are therefore most affected by acute HI (89). Accordingly, the BGT patterns is often seen

in association with an acute sentinel event, such as prolapse of the umbilical cord or placental abruption and is associated with more severe neurodevelopmental impairment (79, 80).

The second pattern (15-45% cases) involves injury in the watershed areas ("WS pattern"), i.e. the vulnerable border areas of the white matter and cortex supplied by the anterior and middle cerebral arteries or the middle and posterior cerebral arteries. Injury extends from the white matter to the overlying cortex in the most severe cases. WS injury is generally considered to be related to prolonged partial HI, since in these instances blood is redistributed to the regions with high metabolic demand (e.g. BGT). Newborns with WS injury tend to have normal motor outcomes in early childhood, however they are at risk of cognitive and language impairment (80, 90, 91). In clinical practice, a considerable degree of overlap is observed between brain injury patterns (79, 80, 89).

1.2.2.1.2 Periventricular leukomalacia in the preterm newborn

Progress in neuroimaging techniques also greatly benefitted our understanding of preterm white matter injury. In the 1980s, cranial ultrasound could only detect large haemorrhages and the most severe cystic type of white matter injury (cPVL), characterized by focal macroscopic cysts due to tissue necrosis in the white matter around the ventricles (4, 76, 92). This severe form of white matter injury almost inevitably leads to cerebral palsy (93-97). Necrotic white matter injury can also evolve into microscopic glial scars, which may not be visible with traditional ultrasound. These are a more common type of injury and are sufficient to cause a loss in brain volume (76, 98). Necrosis of the periventricular white matter can affect white matter tracts that are rapidly developing at the time of injury, including those connecting the thalamus to the cortex and different regions of the cortex with each other (99).

With the development of MRI techniques, a diffuse type of PVL has increasingly been recognized, characterised by non-focal and non-necrotic disturbances of myelination in the periventricular white matter, typically detected as excessive high signal intensity (DEHSI) on MRI (100). This is thought to be due to diffuse gliosis, with typical neuropathological features including impaired pre-oligodendrocyte development, astrogliosis and microgliosis, and is distinct from the necrotic injury with destruction of all cellular elements that characterises cPVL (101). This diffuse white matter injury is associated with reduced white matter volume, ventricular dilatation and altered white matter microstructure (detected via diffusion tensor imaging) (102-105). Diffuse PVL has emerged as the predominant type of white matter injury, accounting for over 90% PVL cases and occurring in 50% preterm

newborns (106). MRI neuroimaging, alongside diffusion-weighted, diffusion tensor, and susceptibility weighted imaging have also allowed identification of ischemic punctate lesions in the white matter and cerebellum, present in 20% extremely preterm newborns, as well as small intraventricular and cerebellar haemorrhages (107, 108). While rates of the more severe cystic form have declined to less than 5% (below 1% in some centres) with advances in perinatal care, this has not been reflected for the diffuse forms (106, 109-116). The cystic and diffuse forms of white matter injury have been proposed to be different manifestations of an 'encephalopathy of prematurity' (76) as well as distinct pathologies (117). Advances in neuroimaging have also highlighted that injury in the preterm brain is not limited to the white matter but it extends to the deep grey matter, cortex, and cerebellum, all of which contribute to the volume loss (76, 118-120). Moreover, functional changes in brain connectivity occur even in the absence of structural changes, reflecting global differences in brain development following preterm birth, and are associated with neurodevelopmental impairment (121, 122). Crucially, altered functional connectivity in the preterm brain has now been identified during pregnancy using fetal resting-state functional MRI, providing evidence that neurological damage in the preterm newborn starts *in utero* (123).

1.3 Long-term morbidities in the vulnerable newborn: neurodevelopmental impairment

Survivors of perinatal brain injuries are at risk of a range of neurodevelopmental impairments affecting movement, cognition, perception, and behaviour, with potentially lifelong impact (124, 125).

1.3.1 Cerebral palsy and related comorbidities

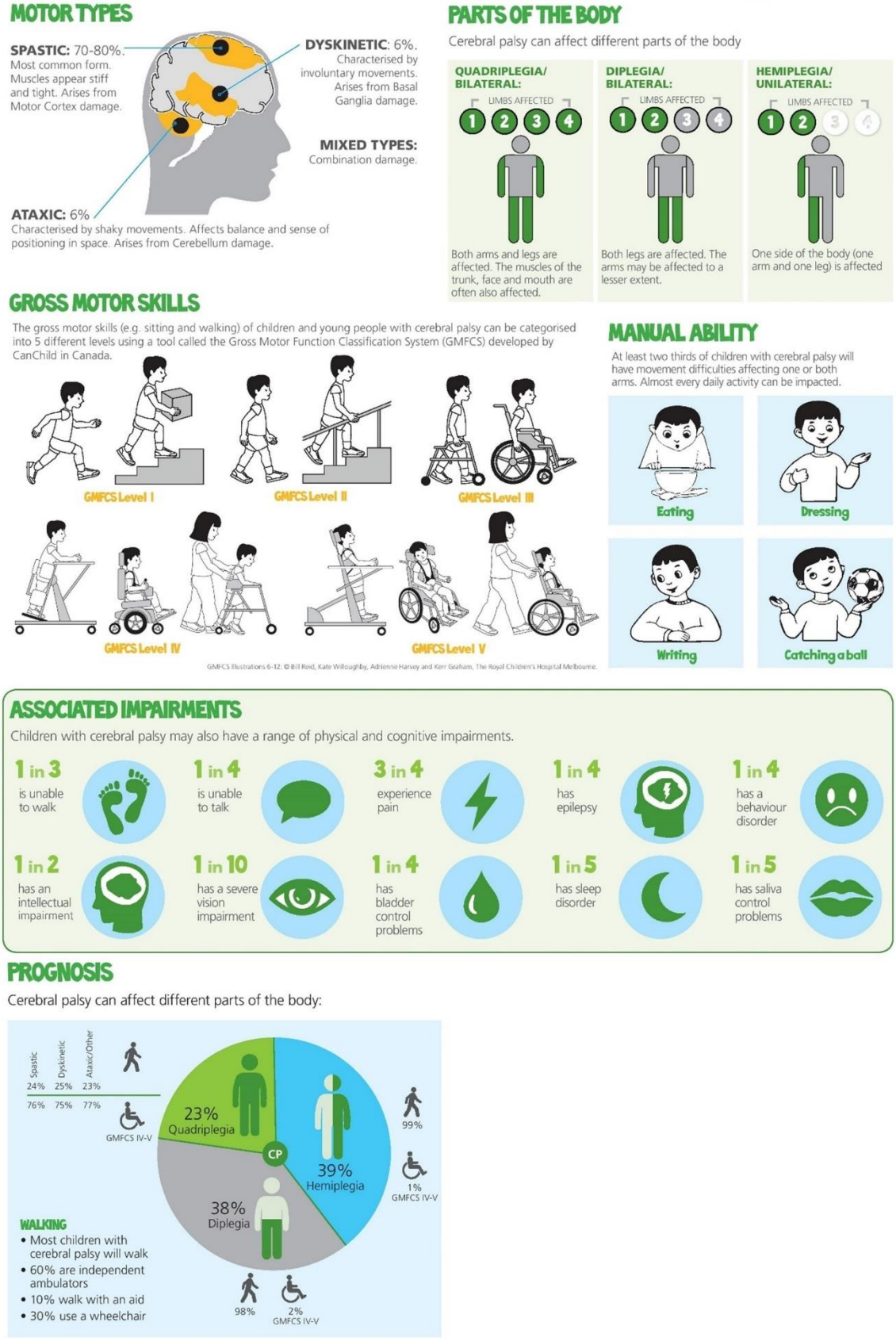
1.3.1.1 Classification of cerebral palsy

While moderately rare in the population, with a prevalence of 1/500 live births, cerebral palsy (CP) is the most common physical disability in childhood in high-income countries, affecting 17 million people globally (126-130). CP is currently defined as "a group of permanent disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour, by epilepsy, and by secondary musculoskeletal problems" (131, 132). CP is an umbrella term assigned at the diagnosis stage by the clinician based on observation of signs and symptoms. It includes

multiple causes and risk factors (many of which are still emerging), neuroimaging patterns and clinical subtypes (133-137).

CP can be classified based on types of motor impairment (spastic, dyskinetic, ataxic and mixed), parts of the body affected (quadriplegia, diplegia, hemiplegia), as well as severity (Figure 1.2) (127, 138). The latter is described by the five grades of the Gross Motor Function Classification System (GMFCS), with grades III to V indicating the inability to walk without a waking aid, including wheelchair use (139). Children with spastic hemiplegia and diplegia represent 75-80% of the total (approximately 40% for each subtype) in high-income countries, with 1-2% requiring a wheelchair. The remaining 20-25% children have quadriplegia (GMFCS grades IV-V), and three quarters require a wheelchair (140). Overall, 40% children with CP are unable to walk independently (10% walk with an aid, 30% use a wheelchair) (140, 141). Evidence from low-income settings is scarce but suggests a higher prevalence of severe forms of CP and additional impairments (142, 143). The most common CP subtypes associated with HIE are spastic quadriplegia and dyskinetic CP (144, 145), those associated with cPVL are spastic diplegia and quadriplegia; vascular causes including haemorrhagic brain injuries and perinatal stroke are typically associated with hemiplegia (106, 137). Importantly, 25-50% of the newborns developing CP have a range of additional neurodevelopmental impairments, including chronic pain, epilepsy, speech impairment, behavioural, sleeping and eating issues, blindness, and deafness (Figure 1.2) (140, 141, 146-148). Notably, approximately half of these children have some degree of cognitive impairment (e.g. IQ, executive function, language). The health complications of CP extend to the entire lifespan and include higher risk of chronic diseases and mortality, likely due to reduced physical activity, poor nutrition, accidents, and poor detection/treatment, as well as lower perceived quality of life for physical functions and social engagement (149-155).

Figure 1.2. Cerebral Palsy infographic (156)



1.3.1.2 Risk factors for cerebral palsy

Since it was first proposed by William Little in 1861 as the consequence of birth asphyxia (157), the definition of CP has continued changing reflecting the evolving understanding of this group of conditions (137). It is now known that HIE is a cause of CP in important but limited proportion of cases, i.e. 10% in high-income countries (2, 61, 145, 158-160). Antenatal risk factors interfering with development throughout pregnancy are estimated to contribute to CP in 70-80% cases, including preterm birth, male sex, maternal infections, placental pathologies leading to intrauterine growth restriction, multiple pregnancies, pre-eclampsia, *in utero* exposure to neurotoxins, post-term birth, congenital malformations and genetics. Postnatal events, such as neonatal infections, strokes, traumatic brain injuries and metabolic disorders make-up the remaining 10-20%, or more in settings with higher infection rates (e.g. in areas where cerebral malaria with febrile seizures is endemic) (129, 161-172). Moreover, the roles of maternal obesity, maternal education, maternal stress during pregnancy, race, ethnicity, and socioeconomic factors are also being explored as risk factors for CP, although disentangling correlation from causation is difficult here since these variables are often correlated to each other and with preterm birth and access to perinatal care (173-189). Overall, it is now recognised that CP results from a combination of multiple predisposing factors and causal pathways more frequently than from a single event (134). Children born preterm represent 30-50% CP diagnoses, making preterm birth a leading cause of CP in high-income settings (172, 190). The risk of CP increases with decreasing gestational age (191), and children born before 33 weeks have 30 times higher risk compared to their peers born at term (192). The current international estimates of CP prevalence by gestational age are 0.1% (1/1,000) live births for children born at term, 0.4-0.7% (4-7/1,000) live births for children born moderately preterm, 3-5% (30-50/1,000) live births for children born very preterm, and 7-10% live births (70-100/1,000) for children born extremely preterm (130, 191, 193-195).

Amongst preterm newborns, the strongest predictor of death or major disability, including disabling CP, blindness and deafness is cPVL (148, 196-199). Up to 90% preterm newborns with CP have some form of white matter injury, whether diffuse or cystic (200). Compared to cPVL, diffuse PVL and punctate lesions are associated with less severe neurological and neurodevelopmental impairment, largely affecting cognitive functioning and risk of psychiatric disorders (102, 201-204), although higher risk of CP has also been reported (199).

Thus, while brain injuries associated with HIE and cPVL are not the only causes of CP, newborns suffering from these two types of brain injuries are very high risk populations: without treatment, 30-40% newborns with HIE (205-211) and 80-100% newborns with cPVL will develop CP (93-96).

1.3.1.3 Other forms of motor impairment

Preterm newborns are 3-4 times more likely to develop deficits in finer motor skills and coordination vs term newborns, with 20% developing moderate impairment and 40% mild to moderate impairment (212-214). Assessing non-CP motor impairment with the Movement Assessment Battery for Children (M-ABC) assessment is essential (215-217), since even mild motor impairment is associated with comorbidities, e.g. cognitive impairment, poorer academic achievement, behavioural problems, and lower social engagement (212, 218-221).

1.3.2 Cognitive and behavioural impairment

1.3.2.1 Preterm newborns as a high risk population

While the majority of preterm children grow up without any difficulties, it is well recognised that the preterm population as a whole is at high risk for a range of cognitive, behavioural, and socio-emotional impairments. Cognitive impairment is indeed the most frequent long-term consequence of preterm brain injury, whether in combination with motor impairment or alone (222). Several international studies have reported a trend for lower cognitive scores with decreasing gestational age and preterm birth to be a risk factor for intellectual disabilities (24, 26, 28, 29, 31, 223-229). Compared to a 1-2% absolute risk of any (mild to severe) cognitive impairment in the general population, very preterm children have 15% risk, rising to 30-40% for extreme preterm children (191, 228, 230, 231). Meta-analyses found that children born very preterm score on average 10-12 points lower (0.7-0.8 standard deviations) than their term born peers, reaching 14-20 points (>1 standard deviation) for children born extremely preterm (232-234). Cognitive and behavioural impairment in late preterm children is less obvious but has nonetheless been reported (235, 236), with a large meta-analysis of 74 studies including over 64,000 children recently confirming a correlation between gestational age and cognitive scores at all preterm gestational ages (237). The study highlighted the extent of impairment, with variation in gestational age accounting for 40% variation in IQ, and differences in IQ between preterm and term children persisting from before to after school. Preterm children also had lower performance in reading, spelling, and maths than their term-born peers, with differences for reading and spelling persisting to secondary school. Additionally, preterm children had doubled risk of attention deficit hyperactivity

disorder (ADHD) (237), alongside an increased risk of autism spectrum disorders that is inversely related to gestational age (238-240). Alongside cognitive impairment, socio-emotional problems (e.g. social competence, self-esteem, emotional regulation) and increased risk of psychiatric disorders (e.g. anxiety, depression, schizophrenia, bipolar disorder) have also been described in very preterm newborns, with proposed mechanisms including interactions between impaired brain development, early life stresses (e.g. pain, maternal stress/depression) and early parental behaviour (241).

1.3.2.2 Risk factors for cognitive impairment

As for motor impairment, cognitive impairment is multifactorial and many of the risk factors overlap. Risk factors include perinatal complications (perinatal brain injuries, preterm birth, neonatal infections, intrauterine growth restriction, bronchopulmonary dysplasia), biological male sex, low socio-economic status, and low maternal education, with the impact of perinatal risk factors diminishing over time as environmental factors (e.g. maternal education, socioeconomic status) gain importance (91, 197, 228, 242-247). The cumulative effect of perinatal risk factors is evidenced by the fact that preterm newborns with multiple risk factors, including intrauterine growth restriction, perinatal infection/inflammation, and socioeconomic disadvantage, have a higher risk of developing executive function deficits at school age (248, 249). Importantly, genetic and epigenetic factors are emerging as important contributors to cognitive impairment and intellectual disabilities (250-252).

cPVL remains the strongest predictor of death or major disability amongst preterm newborns (148, 196, 197), and this includes cognitive impairment (253, 254). Children born preterm with cPVL have on average 10 points lower IQ at school age (255), as well as visuo-spatial impairment (256) and deficits in social cognition (257) compared not only to their term but also to their preterm peers.

Cognitive impairment is a significant problem also in children born at term with HIE. Some degree of cognitive impairment was identified in all trials of therapeutic hypothermia (205-211). Amongst cooled newborns in the NICHD Neonatal Research Network, nearly all the survivors with CP (96%) had moderate to severe cognitive impairment (IQ<70) at school age, as well as a small percentage of those without CP (9%); additionally, about a third of the survivors without CP had milder cognitive delays (IQ 70-84) (243). A systematic review of 7 studies confirmed that even in the absence of CP, 25-60% HIE survivors develop some degree of cognitive impairment by school age (258). Impairment of both general cognitive

outcomes and specific subdomains (executive function, memory, attention, language) is higher for the newborns with moderate to severe HIE (259-261), although even mild HIE may have an effect (260-264). HIE newborns are also at higher risk of behavioural issues, such as hyperactivity and attention problems, which in turn affect educational achievement (258). Importantly, at least 20% of the children identified as having mild or no cognitive impairment still required special educational support (243).

1.4 Clinical challenges: molecular data and precision medicine

1.4.1 Identification, diagnosis, and treatment in childhood

While major disabilities (e.g. severe CP, severe cognitive impairment, epilepsy, blindness, deafness) tend to emerge early in the first years of life, milder forms of impairment might become noticeable only as the child grows to preschool and school age (e.g. milder CP, finer motor skills and coordination problems, milder cognitive impairment, speech impairment, autism spectrum disorders) (140, 141, 146-148). Identification of children affected by neurodisabilities relies on integration of clinical and neuroimaging data from the neonatal period with standardised neurological and neurodevelopmental assessments administered by clinicians during childhood. The majority of neonatal services in England currently offer follow-up until at least 2 years of life (228). A combination of standardised neurological and motor assessments can aid in diagnosis of CP, including the Hammersmith Infant Neurological Examination, the General Movements Assessment, and the Developmental Assessment of Young Children (97, 265, 266). With regard to broader neurodevelopmental assessments at toddler age, the most widely used are the Bayley Scales of Infant Development and the Griffiths Mental Development Scales, which assess strengths and weaknesses in all developmental areas and recommend the most appropriate interventions. As the child grows to preschool and school age, a range of age-adequate tests can be used to assess specific domains, such as the British Ability Scales (BAS)/Differential Ability Scales (DAS), the Kaufmann Assessment Battery for Children (KABC) and the Wechsler Intelligence Scale for Children (WISC-IQ) assessing cognition and basic educational achievement; the Social Skills Rating System and other assessments for social and emotional functioning (267); and the Movement Assessment Battery for Children (M-ABC), assessing fine motor skills, balance and coordination.

The earlier the identification of the brain injury or neurodevelopmental impairment, the earlier the child and family can access a diagnosis and all available neuroprotective strategies both in the short- and long-term (268). The neuroprotective benefit of developmental

strategies during childhood is under investigation. A few studies have assessed the effect of developmental interventions (e.g. physical therapy) specifically in children with CP or cPVL and have mostly suffered from low power or overlap in content of interventions in study and control group (269-271). Most studies have focused on populations at high-risk for neurodevelopmental impairment instead, mostly the preterm population (272, 273). Family-centred programmes that stimulate all aspects of development in high-risk newborns via family coaching have shown clinically meaningful effects in the first 2 years which are attenuated by 5 years (270, 274). Such dissipation of positive effects of home-based developmental intervention was seen in the Avon Premature Infant Project, a prospective study which followed children born very preterm in two Bristol hospitals in the 90s up to 5 years of age (275, 276). In terms of general early developmental interventions (e.g. physiotherapy, speech and language therapy and visual and tactile stimulation), a Cochrane review and meta-analysis reported a significant impact on cognitive and motor outcomes in preterm newborns at toddler age, with cognitive but not motor improvements persisting up to preschool age and neither persisting any further (273). Overall, while more evidence is needed of the long-term effects of developmental interventions, the existing evidence of beneficial effects in early childhood highlights the importance of early identification. Experimental research in developmental neuroscience further supports starting interventions as early as possible, based on evidence of greater plasticity of the brain in the early periods and the existence of sensitive periods, and therefore potential therapeutic windows, for different areas of development (268, 277). Investing in early human development has indeed been proposed as a strategic economic investment for healthcare and society (277).

A change in diagnostic practice has been proposed in the last decade, whereby referral to intervention is not made after a CP diagnosis but as soon as a child is considered at risk (278). This risk-based approach can be extended to all forms of developmental impairment. Notably, a systematic review of reviews recently highlighted that a CP diagnosis can now be accurately made well before the classic 12-24 months period and even before 6 months' corrected age. This can be achieved by combining clinical history with a range of standardised tools including MRI scans (with or without serial ultrasound scans) and assessments of motor skills (General Movements, Hammersmith Infant Neurological Examination, Developmental Assessment of Young Children) (266). Hence, the current translational challenge lies in identifying and integrating as much information as possible

about modifiable and non-modifiable risk factors from before conception all the way to childhood.

1.4.2 Identification, diagnosis, and treatment in the neonatal period

The state-of-the-art NICU represents the first tier of neuroprotection for the vulnerable newborn in the perinatal period, despite also having iatrogenic effects. The real-world impact of perinatal neuroprotective strategies introduced in the last 50-70 years is evidenced by changes in global trends in prevalence of CP and cognitive impairment. Following an initial increase with the introduction of modern NICUs and improved survival (279-281), the prevalence of CP has declined for all gestational ages including extreme preterm newborns (137, 282), and has reached stability since the beginning of the century in Europe (283-286), Australia (193), Canada (287), Japan (288) and China (289, 290), with a slight increase to 3/1,000 live births in the US (291-297). The impact of the improvements in perinatal care and neuroprotection is also evident when considering outcomes of extreme preterm newborns. In 2019, a meta-analysis of 65 cohort studies published between 2000 and 2017 concluded that the improved survival in newborns born between 22 and 27 weeks gestation has been accompanied by a decrease in severe disability (from 36% to 19% for 22-24 weeks, from 14% to 4% for 25-27 weeks) and an increase in disability-free survival (from 1% to 9% for 22-24 weeks, from 41% to 64% for 25-27 weeks) (298).

Two milestones in neuroprotection were achieved in the last decade: the introduction of therapeutic hypothermia for term HIE (≥36 weeks), recommended internationally in 2010 (299-302); and the introduction of magnesium sulphate administered to the mother during preterm labour (<34 weeks) for protection of the preterm brain, recommended internationally in 2015 (303, 304). Meta-analyses show that both therapies reduce risk of CP by approximately a third in their respective populations (303, 305, 306). Currently, these are the only two perinatal interventions with high-quality evidence of effectiveness for prevention of CP, as evidenced by two Cochrane systematic reviews (307, 308).

Currently, 5-10% newborns born before 33 weeks still develop CP and 15-20% develop some form of cognitive and motor impairment (130, 191, 193-195). Moreover, rates of non-CP motor impairment (e.g. developmental coordination disorder) in extreme preterm newborns have nearly doubled (from 23% to 37%) in Australia between 1991 and 2005 (215). For the preterm newborns developing brain injuries, the DRIFT trial (drainage, irrigation, and fibrinolytic therapy) recently reported neuroprotective effects for severe IVH, with evidence

of better cognitive outcomes at 2 and 10 years (309, 310). Crucially, no postnatal treatment for cPVL exists, and the majority of these newborns still inevitably develop motor and cognitive impairment. The highest level of neuroprotection for this population is currently achieved by maximising access to all available interventions for global care of the preterm newborn, which minimise the risk of mortality and severe brain injury (e.g. planned delivery in a tertiary centre, magnesium sulphate, antenatal steroids, prophylactic antibiotics following premature rupture of membranes, supported ventilation) (311). This requires the newborn to be in the right place and the right time, namely tertiary centres with specialised staff, equipment, and medications (312). The impact of being born in the right place has been highlighted by studies of extreme preterm newborns in England (EPICure and National Neonatal Research Database), reporting that delivery in a tertiary centre improved not only the chance of survival but also survival without brain injury and disability (47, 313). A key challenge for the preterm population is early identification of newborns at risk and appropriate planning.

With regard to HIE, six international trials of hypothermia between 2005 and 2013 have shown a reduction in the rate of death or neurodisability by 2 years to 40-50% and the rate of CP to 20-30% (205-211). While it is still too early to evaluate changes in the HIE population in the post-hypothermia era, there are some encouraging findings suggesting a trend for reduced incidence of CP in Sweden, a further drop in rate of death or severe disability to 29% in the US (314) and a reduction in CP severity in the UK (145, 282, 315). However, approximately 30% cooled newborns with HIE still die or develop a severe disability, and approximately 20% still develop CP (314).

A main issue with hypothermia treatment is missing the 6h-therapeutic window (316, 317). Hypothermia treatment requires rapid access to specialised equipment for precise temperature control and MRI imaging, which tend to be present mostly in tertiary units. Therefore, a key challenge not only for preterm but also term newborns lies in shifting away from a “wait-and-see” approach where decisions have to be made rapidly based on the clinical course around the time of birth, and access to specialised care may not be possible within the short time frame. Another key issue is that not all newborns that are treated benefit from hypothermia (number needed to treat = 7 (95% CI 5-10)) (306). Treatment may be less or not effective if brain injury is too severe (89), and in newborns with different pathologies including neonatal encephalopathy with intermittent or prolonged hypoxia during pregnancy (317) or misdiagnosed conditions mimicking HIE (e.g. congenital anomalies, neuromuscular

conditions and sepsis) (89). To the opposite end, newborns with mild HIE do not currently qualify for hypothermia, however 20-30% go on to develop abnormal neurological outcomes and it is not clear whether they may also benefit from cooling and further stratification at the diagnosis stage (262, 318-322). A key challenge for the term population is refining and improving rapidity and accuracy of HIE diagnoses.

Currently, HIE diagnosis is based on integration of multiple types of evidence, in order to distinguish intrapartum hypoxic-ischemic causation from unspecific signs of foetal distress (2, 3):

- Biochemical evidence (in the delivery room/NICU): metabolic acidosis in intrapartum cord blood/early neonatal blood due to impaired gas exchange in the placenta
- Clinical evidence
    - (in the delivery room): sentinel intrapartum events, sudden and sustained bradycardia, low Apgar score at birth, need for prolonged resuscitation
    - (in the NICU): neurological examination with the Sarnat method (e.g. assessing seizures, altered consciousness, altered tone and reflexes, inability to start or maintain breathing and sucking, multiorgan dysfunction)
    - (childhood follow-up): later diagnosis of spastic quadriplegia or dyskinetic CP
- Electrophysiological evidence (in the NICU): discontinuous or flat trace and/or seizure activity on amplitude-integrated electroencephalography (aEEG)
- Neuroimaging evidence of brain injury (in specialist facilities): MRI in the hours after birth

All of the available biomarkers have some limitations. For example, coupling the Sarnat neurological examination with aEEG enhances predictive power, however this is lost during hypothermia (323, 324). Magnetic resonance biomarkers in the neonatal period are currently the best predictors of long-term neurodevelopmental outcomes and are not affected by hypothermia (325-331). These include MRI measurements (e.g. injury in the BGT, PLIC, ALIC and watershed) (329, 330, 332, 333), and MRS measurements (e.g. concentration of neuronal N-acetyl aspartate (NAA) in the thalamus; Lac/NAA peak-area ratio) (320, 334, 335). Conventional MRI carried out around 4-6 days has 90% sensitivity (ability to identify true positives), however this improves after the first week of life as injury develops; moreover, specificity (ability to identify true negatives) is relatively low (around 50%). MRS outcomes reflecting oxidative metabolism in the deep grey matter have higher specificity

(around 95%) but lower sensitivity (around 82%) (326). Both require advanced equipment which is not always available within the required time frame.

In conclusion, the current clinical challenges for both preterm and term brain injuries lie in the need for novel neuroprotective interventions, as well as earlier, more rapid, and more accurate identification of newborns at risk. Integration of molecular data (e.g. genomic, epigenomic, transcriptomic and proteomic data) has the potential to partly address these clinical challenges.

1.4.3 Integration of molecular data may assist with earlier risk stratification and more tailored treatment

The molecular field has been developing rapidly in the last two decades, building on the sequencing of the human genome in 2003 and the substantial technological advances that have followed, which have made these tools increasingly time- and cost-effective. Molecular tools currently used primarily within research settings are hoped to become increasingly adopted in clinical practice in the future. Genetic variation amongst individuals is emerging as an important contributor to risk in complex multifactorial diseases under the influence of both genes and environment. Newborn brain injuries are multifactorial, with a central role of environmental insults, such as hypoxia-ischaemia, infections, and developmental immaturity due to preterm birth (see 1.5). Their genetic architecture is largely unknown, but the common observation that some newborns fare better than others upon being exposed to similar insults suggests that individual response to such environmental stressors and therefore vulnerability to injury may have a genetic component. Moreover, the genetic basis of neurodevelopmental impairments that develop during childhood is also emerging (see 1.5.4). In theory, genetic risk could be assessed early in pregnancy and provide a layer of information available to the family and the neonatologist well before birth and the neonatal stay in the NICU. This has the potential to promote a shift towards earlier identification of high-risk pregnancies and creation of a tailored care plan that maximises the possibility to intervene on the modifiable components of risk at the most appropriate time in the most appropriate place. Extending the scope of available information which can be offered by the clinician could also greatly help mothers and families, who have increasing control over many choices around birth and often little prior objective evidence to support them in the decision-making process (336, 337). A better understanding of genetic risk may be particularly relevant for cPVL, for which no postnatal treatment exists.

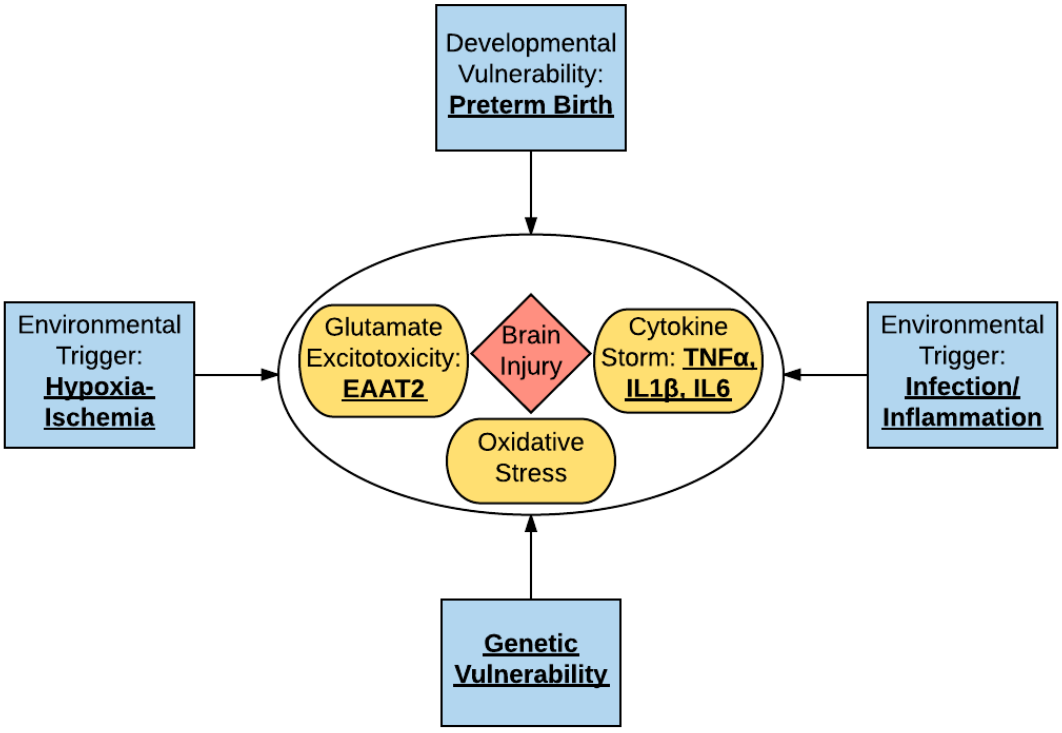
The translational value of molecular tools goes beyond assessment of the fixed component of risk held within the DNA and extends into exploration of how genes important for brain function are dynamically (dys)regulated in response to environmental stressors in their journey from DNA to mRNA to proteins. Exploring gene regulation can not only give insights into the pathophysiology of injury, but also support candidacy of therapeutic targets and molecular biomarkers for rapid diagnosis and risk stratification at birth. This is particularly relevant for HIE. The underlying concept is that the acute hypoxic-ischemic insult triggering HIE will cause changes in expression of many genes in the brain, some of which will have key roles in the injury process. This will lead to measurable changes in gene expression in the brain, which may correlate with measurements in easily accessible peripheral tissues at birth (e.g. cord blood, early neonatal blood, cerebrospinal fluid) that are often collected during routine care. So far, research has focused on measuring serum levels of proteins from genes thought to participate in the pathophysiology of injury (e.g. neuronal injury, astrocyte injury, neuroinflammation, vascular and blood brain barrier damage, oxidative stress, and metabolic intermediates) (see 1.5) (338-347). Clinical studies of HIE biomarkers have largely been small, single-centre studies with variable measurement timings and outcomes (344), and no single circulating biomarker has yet been identified in this relatively new field of research. A type of molecular biomarker which awaits exploration is represented by epigenetic marks, which may be laid on DNA following environmental stresses (e.g. HI) and potentially disrupt gene regulation both in the short- and long-term, acting as a molecular memory (see 1.5.5). Assessing epigenetic biomarkers has the potential not only to improve diagnosis at birth but also prediction of long-term outcomes and, more broadly, the understanding of the pathogenesis of HIE.

1.5 Pathogenesis of perinatal brain injuries

The aetiology of perinatal brain injuries is multifactorial, including environmental and genetic factors, maternal and fetal/neonatal factors, antenatal, intrapartum, and postnatal factors, all of which are likely to contribute to short- and long-term outcomes. Half a century of research into the mechanisms of perinatal brain injuries has consistently pointed to two main upstream triggers, infection/inflammation, and hypoxia-ischaemia (77, 98, 106, 117, 348, 349). These insults are thought to interact in the vulnerable immature brain and converge onto three downstream mechanisms of injury: excessive neuroinflammation and glutamate excitotoxicity, which interact and potentiate each other in a bidirectional manner (see 1.5.3.3), ultimately leading to free radical attack, which directly damages cell components as

well as triggering delayed cell death by apoptosis. Severity and temporal profile of hypoxia-ischaemia (HI) and infection/inflammation (I/I), degree of brain maturity (i.e. gestational age), comorbidities, biological sex and genetic background may all contribute to individual differences in susceptibility to injury, pathogenesis, and clinical presentation. Thus, brain injuries are thought to be induced by complex interactions between environmental triggers - cerebral hypoxia-ischaemia and infection/inflammation - developmental and genetic vulnerabilities. Disentangling these mechanisms will be key for developing clinical strategies aimed at prevention and treatment (101, 350-353). This project focuses on candidate genes thought to be involved in the responses to hypoxia-ischaemia and inflammation, namely the main glutamate transporter (*EAAT2*) and three key pro-inflammatory cytokines (*TNFα*, *IL1β* and *IL6*), as described below (Figure 1.3).

Figure 1.3. Diagram of the proposed pathogenetic mechanisms of newborn brain injuries and candidate genes examined in this project



1.5.1 Developmental vulnerability: preterm birth

The brain undergoes rapid and critical developmental events during the peak time of premature brain injury (24-32 weeks), including neuronal migration and proliferation, growth of axons and dendrites, synaptogenesis, development of the vascular system and myelination. Interference with these trajectories may contribute to selective vulnerability of brain cells and

regions and alter subsequent development. Preterm birth itself induced in the baboon by elective delivery in the absence of exacerbating factors (e.g. infections, growth restrictions) causes subtle brain injury (e.g. white matter gliosis, ventricular dilatation, loss of neurons in the hippocampus, and subarachnoid and intraventricular haemorrhages), with the extent of injury correlating with the regimen of mechanical ventilation (55, 354).

While neuronal loss in the cortex and deep grey matter is frequently seen in the term newborn, in the preterm brain the pre-oligodendrocyte and the periventricular white matter are the most vulnerable cellular and regional targets, leading to the classic neuropathology of PVL (98, 101). Amongst their functions, oligodendrocytes are responsible for laying the highly specialized myelin membrane around axons and are therefore key for development of the white matter. Myelination begins before birth and peaks in the first two years of postnatal life, with the intracortical fibres of the cortex being myelinated well into the third decade. The process of myelination requires that oligodendrocytes first proliferate and develop into mature oligodendrocytes and then depose myelin around axons (106). Around the peak time of preterm brain injury (28-32 weeks of gestation), the pre-oligodendrocyte stage still represents the majority of the oligodendrial pool in the very preterm brain (355, 356). Pre-oligodendrocytes are more vulnerable than mature oligodendrocytes to hypoxia-ischaemia, infection/inflammation, oxidative damage and ultimately cell death (98, 109, 357-363). Indeed, a unique feature of diffuse periventricular white matter injury is an arrest in the development of oligodendrocytes at the pre-oligodendrocyte stage, leading to the abnormal myelination patterns typically seen through MRI (98, 109). In more severe cPVL, necrotic injury extends to all cell components leading to cysts and focal axonal degeneration which exacerbates myelin injury (77, 364). Certain neuronal populations are also particularly vulnerable in the preterm brain, including subplate neurons. Around the peak time for cPVL, these neurons send axons to promote and support development of the cortex before the new axons projecting from the thalamus, corpus callosum and some regions of the cortex enter the cortex itself. Moreover, around this time, GABAergic interneurons migrate to the cortex radially from the dorsal telencephalic subventricular zone and tangentially from the ventral germinative epithelium of the ganglionic eminence and may therefore find themselves travelling through a minefield of activated microglia and astroglia (348, 365). The notion that the neonatal brain responds to injury in a way that is dependent on developmental age is supported by evidence that moderately preterm newborns (32-35 weeks) and full-term newborns with intrapartum HI have different neuromaging and clinical pictures, with the

former having a higher incidence of white matter injury and IVH, as well as a higher incidence of spastic diplegia (160).

Concurrent developmental vulnerabilities affecting preterm newborns include the inability of the immature brain to synthesize appropriate amounts of growth factors needed for brain development and endogenous neuroprotection, as well as an immature immune system (366). Indeed, preterm newborns have reduced numbers of monocytes and neutrophils and reduced ability of these cells to fight infections, as well as lower levels of cytokines; on the other hand, perinatal challenges to the immune system can promote an excessive and sustained inflammatory response, inducing immune tolerance and reducing immune function in the newborn (53). The specific developmental events affecting the immature immune system and increasing vulnerability of preterm newborns to infection/inflammation are still largely unknown and focus of current research (367). The immature brain is also particularly vulnerable to oxidative stress and hypoxia due to lower antioxidant capability, higher content of fatty acids vulnerable to lipid peroxidation, and higher content in iron, which is essential for growth but can also contribute to free radical generation (368). Overall, it is becoming increasingly evident that, aside from the most severe injuries causing necrotic cell death and cysts, much of the chronic disability in survivors of preterm birth stems from impaired development of brain cells at a critical time for brain growth and establishment of brain connectivity (369). This dysmaturation is likely to be multifactorial, with main contributors being hypoxia-ischaemia and infection/inflammation, including that triggered by ventilation support (370, 371).

1.5.2 Upstream environmental triggers

1.5.2.1 Perinatal infection/inflammation

Since the first studies reporting an association between inflammation, brain injury and CP in the 1990s, an active area of research has emerged exploring the role of infection/inflammation in newborn brain injuries and neurodevelopment (372-374). Currently, a substantial body of epidemiological, neuropathological, and experimental evidence supports a causal relationship between local and systemic inflammation and white matter injury (and potentially grey matter injury) in the preterm newborn (375-382). Substantial evidence also exists that exposure to inflammation sensitises the term brain to hypoxic-ischemic brain injury, contributing to HIE (383, 384).

Exposure to inflammation can occur across the perinatal period, from the fetal stage (e.g. maternal intrauterine infections), to labour and childbirth (e.g. early neonatal sepsis), to the neonatal period (e.g. late neonatal sepsis, inflammatory comorbidities such as necrotising enterocolitis) (53, 375-379, 382, 385-395).

Maternal genitourinary infections during pregnancy can trigger an inflammatory response in the foetus leading to fetal inflammatory response syndrome (FIRS). This syndrome increases risk of perinatal death and neurological, pulmonary, and cardiovascular morbidities (396-402), including CP and cognitive impairment (387, 403-405). Chorioamnionitis is the most common antenatal infection and involves a bacterial infection of the placenta arising during prolonged labour or premature rupture of membranes. It is the most common cause of spontaneous preterm birth, causing up to half of spontaneous preterm births (406-409), and is associated with substantial mortality and morbidity (397, 410-413). Clinical studies have reported an association with CP in both preterm and term newborns, with the strongest association seen in the preterm newborn; indeed, the National Institute for Health and Clinical Excellence (NICE) guidelines state it as an independent perinatal risk factor for CP at all gestational ages (387, 389, 390, 392, 393, 401, 414-419). There is substantial evidence that chorioamnionitis is specifically associated with preterm brain injury, including both severe IVH and cPVL (382, 388, 389, 391, 397, 398, 401, 410, 420-428). There is also some evidence that chorioamnionitis and maternal fever are associated with neonatal encephalopathy in term newborns (429-432). While the FIRS is often seen in association with chorioamnionitis, it may also be triggered by maternal blood-borne or respiratory infections. Common parasitic (toxoplasmosis) and viral (rubella, cytomegalovirus, herpes simplex) extra-amniotic infections are minor causes of CP in high-income countries, nonetheless they have been shown to increase risk (433). Some studies related to the Covid-19 pandemic have reported that, while the majority of mothers have good outcomes, there may be an increased risk of premature rupture of membranes, preterm birth and fetal distress, alongside the uncommon but possible risk of vertical transmission (434-437). The inflammatory profile and long-term consequences on neurodevelopment in these newborns will have to be followed up closely.

Infection/inflammation can also occur around the time of birth or shortly after. Early onset neonatal sepsis is detected from positive microbiological cultures in blood or cerebrospinal fluid in the first 3 days of life and assumed to originate from transmission during childbirth, while late onset neonatal sepsis is detected after 3 postnatal days and assumed to originate

from the postnatal environment (438). Newborns who develop neonatal sepsis are more likely to have prolonged hospital stays and neonatal complications and higher mortality (439). Preterm newborns are a high-risk population due to the immaturity of their immune system, higher incidence of premature rupture of membrane, prolonged labour, prolonged hospitalisation, prolonged mechanical ventilation, use of invasive procedures, use of antenatal corticosteroids for prevention of respiratory disease (which may be immunosuppressive) and postnatal corticosteroids (which have been shown to have both benefits and adverse effects, including gastrointestinal, blood pressure and growth problems, and neurological abnormalities at high doses) (53, 440, 441). Moreover, the sickest preterm newborns often have comorbidities which potentiate and exacerbate systemic inflammatory states. Necrotising enterocolitis (NEC) is an acute inflammation of the gut, due to the lining of the intestine becoming invaded with bacteria, leading to sepsis and in the worst cases perforation. This devasting condition has proven one of the hardest to eradicate, leading to death in 20-40% cases and approaching totality in the sickest newborns (54, 442, 443). More than 85% of all NEC cases occur in very premature newborns, pointing to the detrimental effect of immaturity, hypoxia-ischaemia, and inflammation not only for the brain and lung, but also for the gut (444, 445). The effect of NEC on neurodevelopment has been highlighted by a recent systematic review and meta-analysis, which reported that NEC survivors have higher risk of both IVH and PVL compared to premature newborns without NEC, and a large proportion suffer from neurodevelopmental impairment of which CP is the most common (18%); furthermore, the severity of impairment correlates with the severity of gut injury (446). Several studies have reported a higher risk of white matter injury, as well as neurodevelopmental impairment at 2 years in preterm newborns with neonatal sepsis (386, 405, 428, 447-454). A recent systematic review and meta-analysis reported that very preterm newborns with neonatal sepsis have higher risk of neurodevelopmental impairment, including CP and neurosensory deficits, but no difference in cognitive outcomes (455).

The role of neonatal sepsis in term HIE has been explored less than in preterm newborns. In the hypothermia trials, the incidence of neonatal sepsis was relatively high (5-14%) and likely to be higher in low-income settings (306). A population-based study from South Asia reported higher mortality in newborns with encephalopathy also exposed to premature rupture of membranes, which is a known risk factor for early neonatal sepsis (456). Presence of any perinatal inflammatory marker (including maternal fever in labour, clinical chorioamnionitis, fetal tachycardia, prolonged rupture of membranes, early neonatal sepsis,

toxoplasmosis, rubella, cytomegalovirus and herpes simplex infection) was found in approximately a quarter of over 4,000 term newborns in the Vermont Oxford Network Neonatal Encephalopathy Registry (457). A Canadian registry-based cohort studies of children with CP found that, while neonatal sepsis was more prevalent in the preterm population, term-born children with neonatal sepsis were more likely to have more severe motor impairment (spastic quadriplegia and grades IV to V on the Gross Motor Function Classification System) (458).

1.5.2.2 Perinatal hypoxia-ischaemia

The temporal profile of hypoxic-ischemic events is largely different in term and preterm newborns. In term newborns with HIE, defined and acute hypoxic-ischemic events during problematic labour or childbirth (e.g. placental abruption, cord occlusion, uterine rupture, and shoulder dystocia) are usually recognized by the clinician and represent the first step of a diagnosis of HIE, aided by objective clinical and neuroimaging criteria. In neonatal encephalopathy, an acute hypoxic-ischemic event may act as a second hit on top of an existing hypoxic-ischemic condition (e.g. placental abnormalities) with a temporal profile that is more difficult to identify.

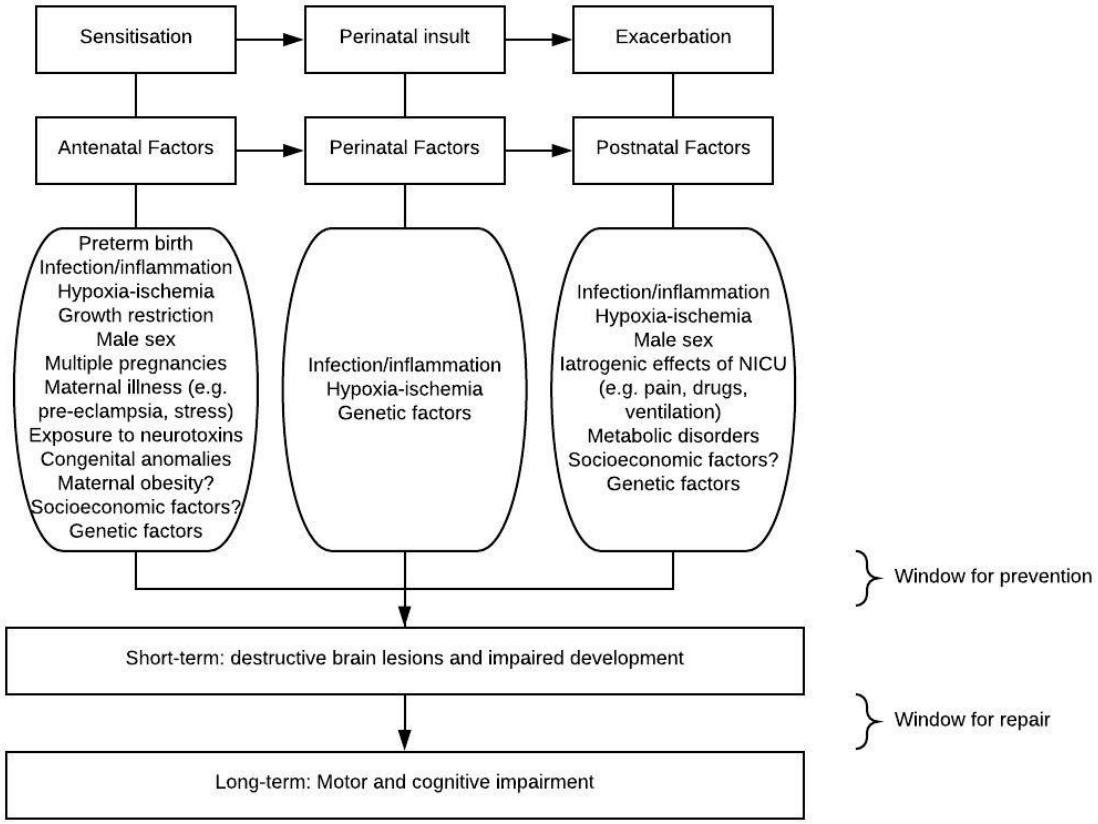
In the preterm newborn, a sentinel event is rarely recognized, and hypoxia-ischaemia is generally assumed to have a more complex temporal profile, with intermittent or chronic nature (364, 459). The foetal brain is adapted for the relatively hypoxic uterine environment and is able to compensate and maintain oxygen delivery via changes in cerebral blood flow, however beyond a certain threshold cerebral hypoxia-ischaemia can occur (460). Short episodes of acute hypoxia can induce labour and preterm birth itself (460). Chronic milder hypoxia due to placental insufficiencies can cause growth restriction, accompanied by structural and functional brain injuries (55). However, it remains challenging to determine the individual contribution of hypoxia-ischaemia amongst several coexistent factors, such as infection/inflammation, growth restriction or hyperoxia due to mechanical ventilation (461). Physiologically, it is conceivable that the preterm brain is vulnerable to hypoxia-ischaemia due to the anatomical and functional immaturity of the periventricular vasculature, which would make the periventricular white matter vulnerable to minor drops in cerebral perfusion (106, 364, 462-468). The periventricular white matter has lower basal blood flow compared to grey matter regions in both humans (469, 470) and the preterm fetal sheep (471-473). Further drops in blood flow are common in sick premature infants with respiratory disease due to lung immaturity (474). Mechanical ventilation may also contribute to ischaemia due to

the vasoconstrictive effect of the induced cumulative hypocarbia (475). A meta-analysis recently reported an association between preterm brain injury and perinatal risk factors related to hypoxia-ischaemia, including oligohydramnios, acidaemia, low Apgar scores, apnoea, respiratory distress syndrome and seizures (476). However, the link between regional differences in blood flow and vulnerability to severe white matter injury is not consistent and, even in moderate ischaemia, some regions of white matter are spared. This suggests that ischaemia is necessary but not sufficient in isolation (77, 352, 472). Indeed, it has been suggested that more consistent evidence is needed to ascertain the specific role of hypoxic and ischemic events in preterm brain injury altogether, and that future research should take into account contributions and interactions with other biological processes, including infection/inflammation and developmental vulnerability (366, 369, 370).

1.5.2.3 Multiple hits

Several experimental studies have shown that a combination of hypoxia-ischaemia and infection/inflammation leads to worse brain injury, gross and fine motor impairment, as well as memory, learning, and behavioural impairment (477-484). Importantly, mild insults that are individually insufficient to cause injury can lead to brain injury when combined (383, 485-493). In the multiple hit paradigm, hypoxia-ischaemia and neuroinflammation interact and potentiate each other in a bidirectional manner, sensitising the brain to subsequent insults and contributing to brain injury (369, 375, 383, 485, 486, 494-496). This is reflected by clinical evidence that multiple perinatal inflammatory and hypoxic-ischemic risk factors (e.g. antenatal or postnatal infection/inflammation, pre-eclampsia, placental pathologies, growth restriction, complicated neonatal course) cumulatively increase risk of brain injury and neurodisability compared to single hits (61, 121, 417, 497-501). The immature neonatal brain is particularly vulnerable to the detrimental effect of these multiple inflammatory and excitotoxic hits, as evidenced by the fact that mild intrauterine infection/inflammation confers protection to subsequent hypoxia-ischaemia in the adult brain but exacerbates injury in the neonatal mouse brain (479).

Figure 1.4. Multiple hit hypothesis for brain injuries in the vulnerable newborn (adapted from (494, 502))



1.5.3 Downstream mechanisms

1.5.3.1 Neuroinflammation and systemic inflammation: focus on TNFα, IL1β and IL6

1.5.3.1.1 Infection/inflammation, brain injuries and pro-inflammatory cytokines

Experimental evidence supports a causal role of infection/inflammation in brain injury. As a standalone insult, infection/inflammation has been studied largely in the context of preterm brain injury, with evidence supporting the developing oligodendrocyte as a key cellular target (494). An acute and severe intrauterine inflammatory insult, caused by injecting bacteria or bacterial endotoxin lipopolysaccharide (LPS, which crosses the placenta) in pregnant animals, causes a fetal inflammatory response, leading to white matter injury and behavioural abnormalities in mice, rats, rabbits, sheep, and macaques (495, 503-509). Chronic intrauterine inflammation also causes a fetal inflammatory response, leading to milder non-cystic injury, nonetheless involving axonal disruption, astrogliosis and remodelling of the cerebral vasculature in the preterm sheep (510, 511). A more recently developed mouse model with intraperitoneal injection of IL1β in the preterm-equivalent pup has shown that even moderate systemic inflammation, which is likely more relevant to the preterm newborn, is capable of

altering the developmental programmes of the white matter. The insult is sufficient to produce myelination defects, with reduced number and diameter of myelinated neurons, partial blockade of oligodendrocytes at the non-myelinating pre-oligodendrocyte stage, altered expression of relevant transcription factors, neuroimaging, and behavioural abnormalities (494, 512). Infection/inflammation has also been explored within a multiple-hits paradigm in the context of HIE (see 1.5.3.3).

Systemic inflammation caused by perinatal infections or inflammatory comorbidities can propagate to the brain via infiltration of peripheral immune cells through the disrupted blood brain barrier and local recruitment of peripheral immune cells (494, 513). Moreover, peripheral cytokines can stimulate vascular endothelial cells in the brain to release pro-inflammatory prostaglandins in the brain parenchyma, which in turn activate microglia and trigger local inflammatory responses (514, 515). Neuroinflammation with glial activation and increase in pro-inflammatory cytokines can also be triggered locally as a result of hypoxic-ischemic injury of brain cells, especially the developing neuron and oligodendrocyte in the term and preterm brain respectively (375, 516). Local and systemic stresses can therefore converge on neuroinflammation and cytokines are key molecular mediators at the core of these processes.

Cytokines are emerging as potential therapeutic targets as well as early biomarkers of injury in both preterm and term brain injuries, since different perinatal stressors (e.g. perinatal infection/inflammation, hypoxia-ischaemia, intrauterine growth restriction, coagulation disorders) appear to converge on their dysregulation (381, 517-520). These soluble proteins link the nervous system to the endocrine and immune systems, and include interleukins, interferons, tumour necrosis factors, chemokines and colony-stimulating factors (521). Cytokines are essential for brain homeostasis and development, contributing to neuroprotective inflammatory responses, synaptic plasticity, learning and memory, and are generally classified into anti- and pro-inflammatory based on their function (522-525). Preterm birth itself is associated with a systemic inflammatory status, with elevated cytokine levels in cord blood and cerebrospinal fluid (CSF) able to differentiate preterm and term newborns (520, 526-529). However, the inflammatory mechanisms relevant to brain injuries are at least in part divergent from those leading to preterm birth, as demonstrated by the fact that intrauterine inflammation insufficient to cause preterm birth can still induce brain injury; moreover, blocking the IL1 receptor prevents brain injury but not preterm birth in mice (530, 531). There are multiple mechanisms through which inflammation may cause brain injury,

including disruption of cerebral blood flow, direct cellular damage mediated by cytotoxic pro-inflammatory cytokines and potentiation of glutamate excitotoxicity (101, 532-534). For example, TNFα has been proposed to mediate injury via multiple mechanisms: by promoting activation of blood coagulators leading to thrombosis and necrosis (535); by increasing permeability of the blood brain barrier (536, 537); by direct damage to myelin and developing oligodendrocytes (538-542); by activating glial cells and propagating neuroinflammation via induction of other cytokines (543); by enhancing glutamate excitotoxicity (544), ultimately leading to oxidative stress and damage to immature oligodendrocytes and neurons (365, 401, 545, 546); and by affecting blood flow. Indeed, TNFα can alter contractility and diameter of cerebral arterioles, with mixed evidence of both vasoconstriction with reduced cerebral blood flow (547-549), and vasodilation with increased cerebral blood flow (550-554) in newborn piglets, adult rats and rabbits. The underlying mechanisms are still unclear and include both endothelium-dependent (e.g. induction of nitric oxide synthase, direct modulation of intracellular Ca^{2+} signals in smooth muscle cells, and endothelium-independent (e.g. ceramide signalling) effects (550-554).

1.5.3.1.2 Experimental evidence supporting candidacy of pro-inflammatory cytokines

Experimental studies support a key role of neuroinflammation in causing brain injury and of these cytokines in being key mediators of this process. Major differences in the inflammatory response following infection/inflammation, hypoxia-ischaemia or a combination of both are seen between preterm- and term-equivalent rats: in the preterm brain, IL1β is the main pro-inflammatory cytokine released and the anti-inflammatory response is absent or very limited; in the term brain, on the other hand, both pro- and anti-inflammatory responses are stronger, including both IL1β and TNFα, as is disruption of the blood brain barrier (517).
Several studies have shown that genetic or pharmacological inhibition of IL1 protects the newborn rodent brain from hypoxic-ischemic injury (555-561), as well as in combined inflammatory and hypoxic-ischemic insults (557, 562, 563). Moreover, knockdown reducing but not eliminating IL1β attenuates injury, interestingly in correlation with upregulation of IL6 (559). IL6 can have pro- or anti-inflammatory functions depending on the pathological context (381, 516, 564). Following HI, it acts as a mediator of inflammation in the acute phase, switching to neurotrophic functions in the subacute and long-term phases (564).

Addition of TNFα to mixed glial cultures or overexpression in transgenic mouse exacerbate injury (565-567). Genetic knockout or pharmacological inhibition of TNFα after an acute HI insult, inflammatory insult or a combined inflammatory/excitotoxic insult are

neuroprotective, reducing brain injury by half in some studies (485, 568-573). In term-equivalent rats exposed to a single inflammatory hit, the *Tnfα* pathway emerged as being associated with cell death/survival in hypothesis-free transcriptome-wide analyses (492). In the preterm foetal sheep challenged by prolonged systemic inflammation, blocking TNFα delays (but does not stop) the rise of IL6 and markedly attenuates white matter gliosis, while also improving recovery of EEG and cardiovascular function (574). TNFα inhibition also normalises total oligodendrocyte count, by reducing the proliferation of pre-oligodendrocytes stimulated by inflammation, without affecting mature oligodendrocytes or myelin expression, though the mechanisms and significance for brain development are still unclear (574). On the other hand, TNFα antibody reduces the elevation of IL1β following neuroinfection (intracerebral injection of LPS) but does not reverse brain injury, suggesting complex dual roles (575). Indeed, mice lacking TNFα receptors are more susceptible to hypoxic-ischemic injury (576).

1.5.3.1.3 Clinical evidence supporting candidacy of pro-inflammatory cytokines

Pro-inflammatory cytokines have been found across the three compartments relevant to the brain-injured newborn (uterus, fetal/neonatal circulation, fetal/neonatal brain) and are able to cross boundaries between them (via the placenta and blood brain barrier) (374). Elevated cytokine levels have been found in the post-mortem brains of preterm newborns with cPVL (in and around necrotic cysts) as well as in ultrasound-defined diffuse PVL across different cell types (including activated microglia, reactive astrocytes, some pre-oligodendrocyte populations, macrophages) (577-584). The role of cytokines in early white matter injury and in less severe MRI-defined diffuse white matter injury remains to be ascertained (101). Post-mortem studies of cytokine levels in term HIE are scarcer but nonetheless include reports of elevated expression (especially of IL1β) in the injured regions (e.g. hippocampus), including in newborns with seizures (585, 586). Moreover, other inflammatory mediators known to regulate these cytokines (e.g. COX2) have been found to be dysregulated in post-mortem HIE brains (586-588).

Cytokines are also found at elevated levels in the systemic circulation of brain-injured newborns. Evidence is substantial for preterm newborns, for whom elevated levels in amniotic fluid, cord blood, early neonatal blood, CSF and urine are associated with PVL, IVH, neurological abnormalities, CP and adverse motor, cognitive, behavioural and educational outcomes (independently of gestational age, birth weight and sex) (249, 374, 589-617). Early cord TNFα is also associated with EEG depression in the newborn period,

which is in turn associated with brain injury and neurodevelopmental impairment (618). Unsurprisingly, preterm newborns with perinatal infections and inflammatory comorbidities (e.g. chorioamnionitis, funisitis, NEC, sepsis, prolonged mechanical ventilation) show particularly elevated cytokine levels in cord and neonatal blood (589, 619-628). Fewer studies failed to find such an association, and suggested explanations include low power (especially for cPVL) and variable time windows for sample drawing and measurement (595, 599, 627, 629, 630). The association between cytokine levels in neonatal blood and long-term neurodevelopmental outcomes is particularly clear in extreme preterm newborns, as shown by the ELGAN studies. In this high-risk population, persistency of inflammation (elevated levels on multiple days in the first two neonatal weeks) and breadth of inflammation (elevated levels of multiple proteins, especially across functional categories) in the first two weeks of life seem to have an important impact on neurodevelopment (605). This is reflected in a 2- to 3-fold risk of CP (e.g. TNFα and TNFα-r1 with diplegia and IL6 with hemiplegia), increased risk of microcephaly, as well as severe motor and cognitive impairment at 2 years (604, 607, 611). There is also a clear association between persistently high levels of 3 or more inflammatory proteins in early neonatal blood and structural neuroimaging findings, including reduced overall brain volume, and reduced volumes for cortex, deep grey matter, cerebellum, and brainstem (631). While smaller cerebellum and brainstem are associated with impaired cognition, the association between inflammation and cognition is not fully explained by reduced brain volumes and other mechanisms are likely to be involved, including impaired functional connectivity, and altered epigenetic regulation of gene expression (631, 632). Indeed, cytokines may affect neurodevelopment in the absence of obvious perinatal brain injuries. This is supported by evidence that the association between persistently high levels in early neonatal blood and attention problems at 2 years is not mediated by ultrasound evidence of white matter injury or early cognitive impairment (612). Interestingly, cytokine levels on day 14 have much higher predictive power than at day 3, which may reflect the accumulation of risk factors after birth (611).

Cytokines are also elevated in the peripheral circulation of term newborns. Children born at term developing CP have higher TNFα, IL1β and IL6 levels in neonatal blood (372). More recently, interest has focused specifically on HIE, with emerging evidence that elevated levels in CSF not only predict HIE but also correlate with HIE severity (346, 633-637), as well as being associated with death or neurological and neurodevelopmental impairment at toddler age (633, 637-639). Cytokine levels in cord blood and early neonatal blood have also

been shown to predict HIE (323, 346, 636, 640-643). This includes association with MRI and aEEG findings (644, 645), MRS-defined impaired oxidative metabolism in the deep grey matter (646) and seizures (647). Once again, elevated levels in blood also predict long-term neurological and neurodevelopmental impairment (323, 639, 640, 646, 648-650) and epilepsy (647).

Importantly, the inflammatory response in these newborns may remain altered in the long-term. Preterm children who had PVL at birth and developed CP maintain an altered inflammatory response at-school age, producing higher levels of TNFα both in plasma and in peripheral blood mononuclear cells following an inflammatory challenge *ex vivo* (LPS stimulation) compared to control children matched by gestational age (651). Similarly, children who had neonatal encephalopathy at birth maintain higher levels of several pro-inflammatory cytokines in serum at school age, both at baseline (including TNFβ, IL6 and IL8) and following LPS stimulation *ex vivo* (including TNFα, IL1β, IL6 and IL8), with elevated TNFβ correlating with low gross motor scores (652). Another study reported that children with CP have higher plasma TNFα, although gestational age was not reported (653). Interestingly, TNFα levels correlated with CP severity measured using the Gross Motor Function Classification System in the subgroups with spastic quadriplegia and diplegia, as well as with degree of improvement after rehabilitation therapy. A study with a Han Chinese sample reported higher plasma IL6 in term-born male adults with spastic CP (654). These findings need to be confirmed by larger studies but suggest that the insults leading to perinatal brain injury might program inflammation to be sustained in the long-term, potentially offering an extended window for intervention. Tertiary mechanisms of injury, mediated by epigenetic modifications, may sustain dysregulation and sensitisation in the long term, interfering with remodelling and repair mechanisms (380, 655).
Amongst pro-inflammatory cytokines, TNFα, IL1β and IL6 are not only the most frequently studied, but also the most predictive of neurodevelopmental outcomes, as reported by two systematic reviews (613, 656). Candidacy of these cytokines is supported by the finding of dysregulation in a range of neurodevelopmental, psychiatric, and neurodegenerative disorders (657-661).

1.5.3.2 Glutamate excitotoxicity: focus on EAAT2

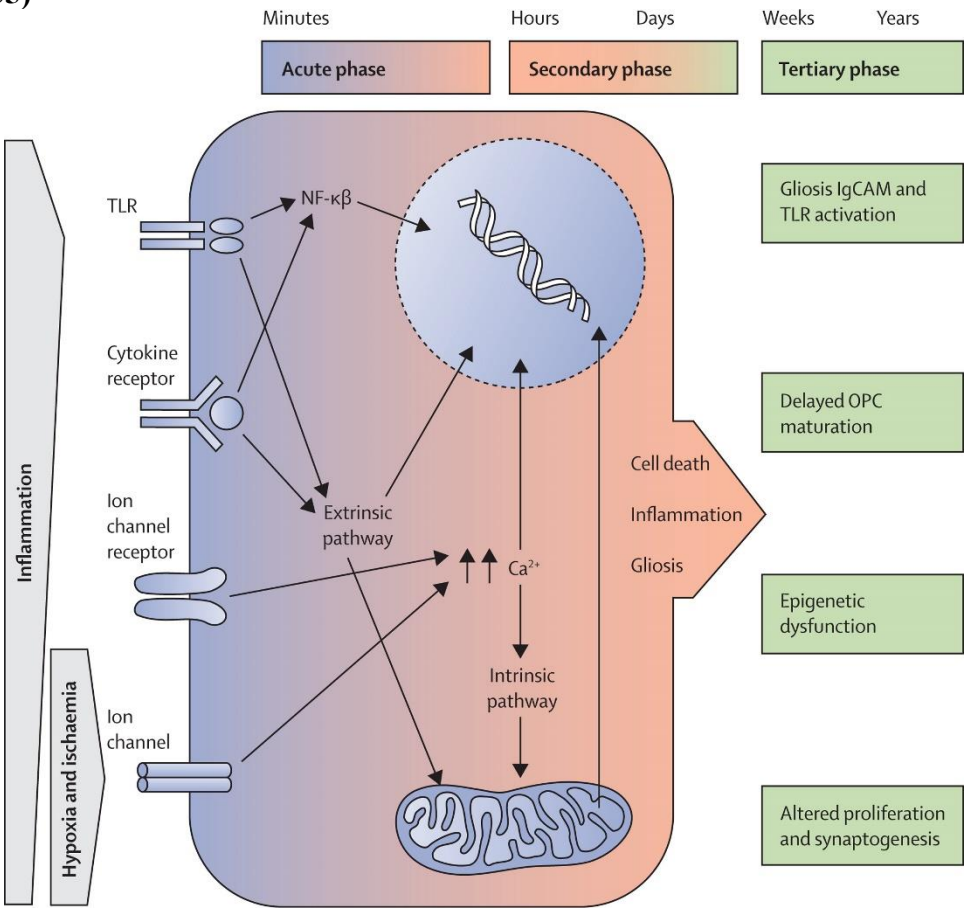
1.5.3.2.1 Hypoxia-ischaemia, glutamate excitotoxicity and brain injuries

An acute HI insult leads to brain injury in first instance via disruption of glutamate homeostasis. Glutamate is the main excitatory neurotransmitter in the mammalian brain

(662). It is essential for brain function, orchestrating not only fast excitatory neurotransmission but also long-lasting neuronal changes necessary for memory, learning and cognition. It is also fundamental during brain development, due to its role in regulating formation and elimination of synapses, neuronal migration, proliferation, and viability. Glutamate is abundant inside brain cells and most neurons and glial cells have glutamate receptors distributed across most cellular elements, highlighting the importance of glutamatergic systems for normal function (663-667). Stimulation of a glutamatergic neuron results in Ca^{2+}-dependent release of glutamate in the synapse by vesicular exocytosis. Extracellular glutamate binds to and activates post-synaptic ionotropic (NMDA, AMPA and kainate receptors) and metabotropic (mGluR) glutamate receptors, stimulating the post-synaptic neurons via Ca^{2+} or Na^{+} influx. This induces intracellular signalling cascades which lead to physiological cellular responses, such as regulation of transcription factors and DNA replication (668, 669). The ubiquity of glutamate is a double-edged sword: when homeostasis is disrupted, glutamate can turn into a potent neurotoxin. If the concentration of glutamate in the extracellular space rises above physiological levels, post-synaptic glutamate receptors are overactivated. This excessive activation, or excitotoxicity, leads to cell death via activation of suicide cell programs (apoptosis) (669, 670). Since it was first proposed in the late 1960s (671), the concept of glutamate excitotoxicity has been implicated in several adult disorders, both acute (e.g. ischemic stroke and traumatic brain injury) and chronic (e.g. amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, major depression and addiction) (672, 673). Consistently, injection of glutamate agonists into the cortex, striatum, and periventricular white matter of newborn rodents, rabbits and kittens produces patterns of perinatal brain injuries similar to those seen in humans (674-680).

The cascade leading to glutamate excitotoxicity following an acute HI event is well described and typically involves an acute phase of injury minutes after the insult, and a delayed phase of injury in the hours and days after reperfusion. In the model of HI and inflammatory brain injuries leading to CP, the tertiary phase is less clear but is thought to span weeks and years after the insult and involve prolonged inflammation, impaired development, impaired repair and remodelling mechanisms and epigenetic dysfunction (89, 344, 655, 681, 682).

Figure 1.5. Primary, secondary and tertiary phases of brain injury leading to cerebral palsy (655)



Following an acute HI insult, a primary energy failure occurs due to blood, oxygen and glucose deprivation, causing failure of oxidative phosphorylation and impairment of ATP production and tissue energy reserves. Astrocytes, with their unique oxidative capacity and ability to upregulate ATP production, are central to maintaining energy metabolism during the first stages of ischaemia (683). If the insult is severe, some cells may die at this early stage via necrosis, due to influx of ions and water, cell swelling and bursting, with all cellular elements dying within hours. This is a key process in the development of white matter cysts in the preterm brain (77).

Upon reperfusion and reoxygenation, glucose use is nearly completely restored, as is mitochondrial respiration and production of high-energy phosphates including ATP (latent phase) (682, 684-686). However, impairment of the ATP-dependent Na^+/K^+ pumps in the early phases leads to loss of the electrochemical gradient across the cell membrane. In surviving neurons, depolarization of the cell membrane activates Ca^{2+} channels in the pre-synaptic terminal, triggering vesicular release of glutamate in the synapse. In astrocytes, HI

leads to a failure in the astrocytic glutamate uptake system, which also relies on Na^+/K^+ gradients. The combination of increased synaptic release and reduced astrocytic uptake leads to accumulation of glutamate in the synaptic space and overactivation of post-synaptic ionotropic and metabotropic glutamate receptors (106). The subsequent intracellular Ca^{2+} flooding triggers activation of phospholipases, endonucleases, proteases, and nitric oxide synthase, with degradation of cellular and extracellular structures, and generation of harmful free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS). Additionally, glutamate leaking outside the synapse activates extra-synaptic NMDA receptors, which, contrarily to the pro-survival action of synaptic NMDA receptors, promotes excitotoxic cell death even further (687). This excitotoxic-oxidative cascade eventually leads to cell damage or death via a continuum of necrosis, apoptosis, and autophagy in the secondary phase of injury hours after the insult (76, 77, 671, 688-694). During necrosis, the cell swells until the membrane is ruptured, the intracellular contents are released and lysed, followed by inflammation and phagocytosis. On the other hand, during apoptosis chromatin is condensed, the cell shrinks while the cell membrane remains intact, mitochondria are damaged, death genes (e.g. *Bax*, *Caspases*, *p53*, *Parp*, C*ytochrome* c, *Bid*) are activated leading to cleavage of DNA at specific sites, and the cells dies without inflammation followed by phagocytosis (101). The role of autophagy, a type of cell death mediated by lysosomes, is less clear, but evidence of its involvement in HI brain injury is emerging (681, 692, 695, 696). The predominant cell death mechanism depends on severity of the insult, sex, cell type and developmental stage of the cell (101, 697). Importantly, the secondary energy failure has been evidenced *in vivo* in newborns by MRS imaging. Specifically, a depletion of high energy phosphates occurs in the brain in the 12h following HI accompanied by a rise in lactate, despite maintenance of cardio-respiratory support (685, 698). This secondary energy failure correlates with adverse outcomes including death, brain injury and disability (699). Therapeutic hypothermia interferes with the secondary injury process, posing the requirement for the short time window (700-703).

The different patterns of HI brain injury in the preterm and term brain may be related to differential cellular and regional vulnerabilities at different stages of development, alongside developmentally-regulated changes in expression of glutamatergic genes (98, 101, 106). The white matter in the rat is more vulnerable to HI at preterm-equivalent age, when pre-oligodendrocytes are predominant, than at term-equivalent age, when mature oligodendrocytes are the major form (359, 704, 705). Glutamate is highly toxic to pre-

oligodendrocytes in cell culture, rapidly leading to cell death via free radical attack (357, 706, 707). Pre-oligodendrocytes are also strikingly more vulnerable than immature neurons in moderate global ischaemia in the preterm fetal sheep (708, 709). Accordingly, the patterns of HI white matter injury seem to be determined primarily by the timing of appearance (710) and spatial distribution (472) of pre-oligodendrocytes rather than severity of ischaemia itself. Immature neurons in the preterm brain are also more vulnerable to excitotoxic injury than in the term brain, since NMDA receptors are developmentally upregulated, more permeable to calcium and less sensitive to magnesium block (711). In physiological conditions, the abundance of glutamate receptors in the white matter is key during early neuronal development, contributing to rapid growth and myelination. However, their abundance also confers increased vulnerability in excitotoxic conditions (712). The selective vulnerability of subplate neurons compared to cortical neurons observed in a preterm model of HI has been suggested to originate from a developmentally-regulated increase in glutamate receptors associated with early maturation of these neurons (713). Similarly, it has been suggested that selective vulnerability of the deep grey matter and sensorimotor cortex in term HIE could be related to peaking NMDA receptor expression in these regions around term time and proximity to developing glutamatergic circuits (88).

Cheung et al (714) suggested that glutamate concentration may be key in determining the pathways of cell death, with higher glutamate concentrations preferentially triggering necrosis and lower concentrations leading to apoptosis. Either way, even transient excess of glutamate can start a number of events that can ultimately cause death or damage of vulnerable cell populations (715).

1.5.3.2.2 Glutamate

1.5.3.2.2.1 Experimental evidence of dysregulation of glutamate in brain injuries

In the near-term rat, an acute HI insult sufficient to cause mild white matter injury is followed by a rise in extracellular glutamate, with oligodendrocytes and axons representing the major cellular sources and astrocytes failing to take up excess glutamate (716). In the near-term foetal sheep, repeated umbilical cord occlusion sufficient to cause periventricular white matter injury is characterised by a delayed rise in extracellular glutamate levels, which correlates with the extent of injury and potentially indicates impaired glutamate transport function and establishment of reverse transport (see 1.5.3.2.3) (717). In the term piglet, an acute global HI insult sufficient to cause brain injury recapitulating HIE is characterised by a biphasic pattern of extracellular glutamate levels in the basal ganglia, with an early increase

in the first 6 hours followed by transient and slight recovery by 12 hours, possibly due to partial compensatory upregulation of glutamate transport; a further increase occurred after a day, possibly through cells bursting due to reperfusion injury and reversal of glutamate transport in the late stages of disease (718).

The central role of glutamate excitotoxicity in the pathophysiology of HI brain injury is demonstrated by the fact that pharmacological blockade of postsynaptic glutamate receptors before or immediately after a HI insult is neuroprotective both in preterm (719, 720) and term (675, 721) brain injuries. Indeed, one of the mechanisms through which therapeutic hypothermia and magnesium sulphate are thought to exert neuroprotection in the term and preterm brain respectively is by preventing excitotoxic damage through NMDA receptor blockade (722, 723). An interesting and underexplored therapeutic target for neuroprotection is represented by glutamate transporters, which are essential to prevent glutamate excitotoxicity in physiological conditions.

1.5.3.2.2.2 Clinical evidence of dysregulation of glutamate homeostasis in brain injuries

Clinical evidence of disruption of glutamate signalling following acute HI is provided by evidence of elevated glutamate levels in CSF and basal ganglia (MRS spectroscopy) of newborns with HIE in the first day of life (724-727). Glutamate levels in CSF can distinguish severe from mild or moderate HIE (725). Moreover, glutamate increase in serum is significant at 24h of life in newborns with HIE, peaks at day 3 and returns to normal by day 7, with serum levels correlating with HIE severity (728, 729). Since glutamate is specific to the brain, it has been suggested as a sensitive biomarker for brain injury (346).

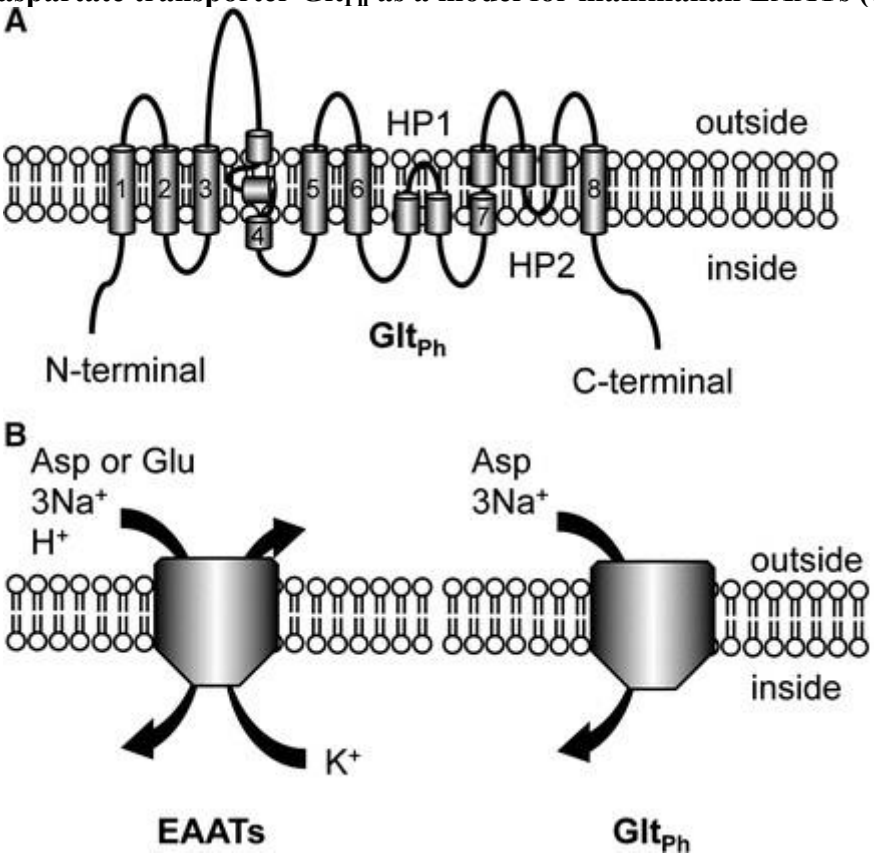
Elevated glutamine levels have been found in MRI-defined punctate necrotic white matter lesions in the preterm brain (730). Glutamate is taken up into astrocytes for conversion into glutamine, which is shuttled back to neurons. The finding of elevated glutamine rather than glutamate may be due at least in part to the temporal lag between insult and measurement. An important limitation of *in vivo* glutamate measurements in preterm newborns is that the peak window of glutamate changes is probably missed because magnetic resonance measurements are likely to be carried out long after the initial insults in newborns that have already become sick. With this in mind, these findings more broadly suggest that disrupted glutamate homeostasis persists in the subacute phase in moderate necrotic white matter injury. Although a relatively small subset of the newborns with punctate lesions also had evidence of cysts, no studies to date have measured glutamatergic metabolism specifically in newborns with cPVL.

1.5.3.2.3 Glutamate transporter

The brain has no known enzymatic mechanism to metabolise glutamate in the extracellular space and it relies on intracellular glutamate uptake by glutamate transporters expressed primarily on the membrane of astrocytes (669, 672, 731-735). Within astrocytes, glutamate is converted to glutamine, which is shuttled back into the pre-synaptic neuron, ready for conversion to glutamate. The glutamate-glutamine cycle is not essential for supplying glutamate for neuronal release but is required for homeostasis of glutamate signalling (669, 672, 736). Astrocytes are in close interaction with neurons: their processes ensheathe synapses and are in direct contact with the brain vasculature, forming organised anatomical domains; moreover, they communicate with neurons via a range of transporters, receptors, ion channels, cytokines and growth factors (737). Given the close interaction with neurons, it is not surprising that astrocytes (the most abundant cell types in the brain) are considered part of a "tripartite synapsis" alongside pre- and post-synaptic neuron terminals, and that disturbances in their function can have catastrophic consequences for neighbouring neurons (738).

The five members of the excitatory amino acid transporters (*EAATs*) family carry out most of the glutamate clearance in the central nervous system (739), especially *EAAT2* (or *SLC1A2*, rodent orthologue *Glt1*) and *EAAT1* (or *SLC1A3*, rodent orthologue *Glast*) (740-742). *EAAT2*/*Glt1* is the major glutamate transporter in the forebrain, except in the cerebellum, circumventricular organs, and retina, where *EAAT1*/*Glast* is prevalent. The structure, function and pharmacological properties of the transporter have been studied via a prokaryotic homolog of the EAATs which transports aspartate in *Pyrococcus horikoshii* (Glt_{Ph}). Thanks to these studies, it was possible to characterise the structure of the mammalian transporters including EAAT2/GLT1 (Figure 1.6). The transporter is localised to the cell membrane and has 8 transmembrane domains, 4 of which make up the central core while the other 4 together with two hairpins form the transport domain (734, 743). Glutamate transport is carried out through an ATP-dependent process driven by Na^+ and K^+ gradients, whereby glutamate and aspartate are co-transported inside brain cells with 3 Na^+ and 1 H^+ for the antiport of 1 K^+ (Figure 1.6). EAAT2 is also a selective anion channel, transporting Cl^- anions during intermediate conformations, uncoupled from the flux of glutamate (743).

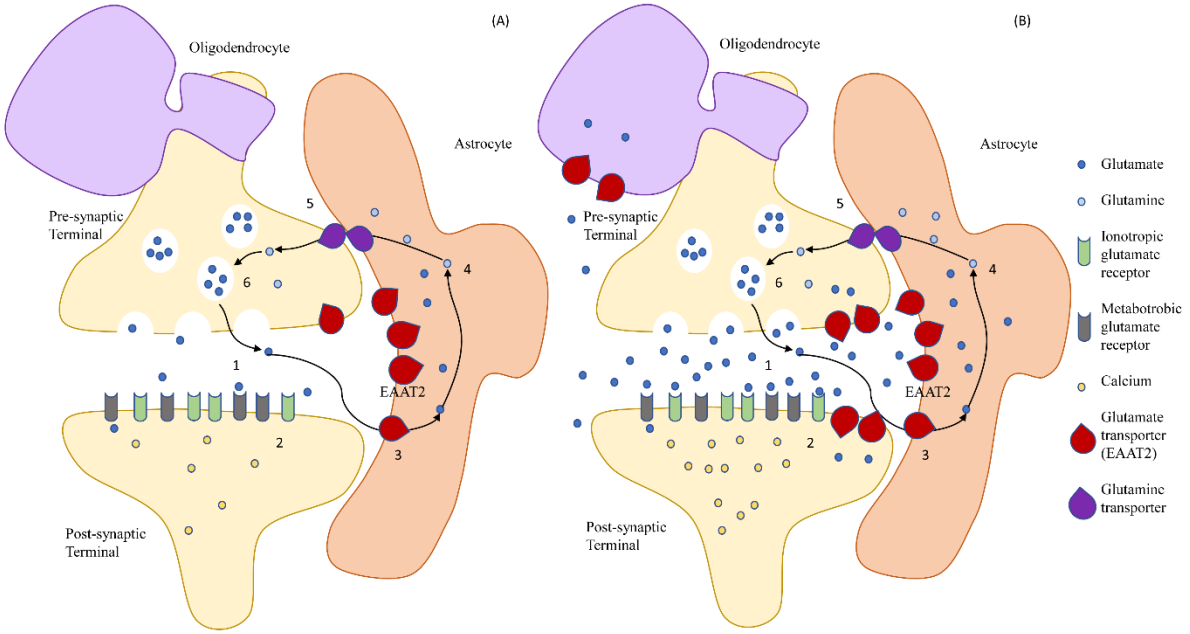
Figure 1.6. a) topology; b) stoichiometry of EAAT2 transport derived from prokaryotic homologue aspartate transporter Glt_{Ph} as a model for mammalian EAATs (672)



EAAT2/*Glt1* is expressed predominantly by astrocytes in the term and adult brain, as well as at lower levels in several neuronal populations at the pre-synaptic axon terminal (e.g. in the rodent hippocampus and somatosensory cortex) (669, 736, 744-747). Importantly, it is transiently upregulated in pre-oligodendrocytes and some neuronal populations (e.g. layer V pyramidal neurons, layer I neurons, interstitial white matter neurons and subplate neurons) during mid-late gestation, at the peak time for preterm brain injury (748, 749) (

Figure 1.7). Accordingly, oligodendrocytes and axons from the rat optic nerve are subject of extensive excitotoxic injury upon experimental inhibition of glutamate transport (750).

Figure 1.7. Glutamate signalling under a) physiological and b) excitotoxic conditions in the immature brain
(A) In the mature healthy brain, glutamate is released by exocytosis from the pre-synaptic neuronal terminal into the synapse (1), where it binds to post-synaptic ionotropic (NMDA, AMPA, kainate) and metabotropic (mGluR) glutamate receptors, inducing Ca^{2+}-mediated signalling cascades that result in cellular responses (2). Extracellular glutamate is taken up primarily by astroglial EAAT2 (3) and converted to glutamine (4), which is shuttled back to the pre-synaptic terminal via glutamine transporters (5). Here, glutamine is converted back to glutamate (6). (B) During excitotoxicity, a combination of increased neuronal release and decreased astroglial uptake leads to a rise in extracellular glutamate levels, overactivation of the post-synaptic glutamate receptors, Ca^{2+} overload, and activation of apoptotic pathways. Reversal of transport of astroglial transporters may also contribute to the accumulation of extracellular glutamate. In the immature brain, upregulation of the glutamate transporters in underdeveloped neurons and oligodendrocytes may contribute to their selective vulnerability



GLT1 may be neurotoxic not only when downregulated leading to reduced glutamate transport, but also when upregulated if reverse transport is established - both mechanisms ultimately leading to increased extracellular glutamate concentrations. Reverse transport has an outward direction and is driven by the transmembrane gradient of excitatory amino acids independently of ATP and Ca^{2+} (668, 751, 752). In this scenario, glutamate transporters become themselves a major source of extracellular glutamate, potentially turning into key contributors of excitotoxic injury (753). Notably, inhibiting reverse GLT1 transport in the rat striatum protects against ischemic cell death (754). Moreover, knockout mice lacking GLT1 are more vulnerable to neuronal death after a short, severe episode of ischaemia than wild-

type mice, suggesting that GLT1 function is essential for neuroprotection when ischaemia is acute; on the other hand, wild-type mice expressing GLT1 are more vulnerable to neuronal death than mice lacking GLT1 during extended, chronic ischaemia, suggesting that GLT1 (via reverse transport) becomes neurotoxic when ischaemia is prolonged (755).

1.5.3.2.3.1 Experimental evidence supporting candidacy of glutamate transporter

Several lines of evidence support the central role of *EAAT2* expression/function in maintaining extracellular glutamate homeostasis. The high concentration (1 mg/g rat brain tissue), ubiquity (1% of total CNS protein in the adult brain), constitutively high expression and high degree of conservation across mammalian species are all indications of the physiological importance of *EAAT2*/*Glt1* (669, 736, 743, 756). Importantly, selective overexpression of GLT1 in astrocytes is neuroprotective during ischaemia (757). On the other hand, pharmacological inhibition of glutamate transport, including EAAT2/GLT1, leads to rapid extracellular glutamate increase *in vitro* (758) and overactivation of post-synaptic NMDA receptors (759). Genetic deletion via constitutive knockout in the mouse leads to lower body weight, seizures, acute cortical injury in the forebrain and increased mortality from the second/third postnatal week (760). This delay reflects the dramatic developmental upregulation that occurs from the second postnatal week throughout the brain (especially in the cortex, striatum, caudate nucleus, and hippocampus) (761-766). Brain tissue from knockout mice lacking GLT1 shows much lower (5%) glutamate transport activity compared to wild-type, suggesting that GLT1 is responsible for up to 95% of glutamate transport. This is confirmed by the ability of GLT1 antibodies to remove 90% of the transport activity in forebrain tissue (767). Other GLT1 knockouts have confirmed the obvious phenotype, with lower life span, lower body and brain weight, mild loss of CA1 neurons in the hippocampus, severe focal neuronal loss in layer II of the neocortex and focal gliosis (768). A conditional knockout mouse with selective deletion of GLT1 reproduces this phenotype while ruling out developmental adaptations (769). Inhibition with antisense oligonucleotides *in vitro* and *in vivo* induces a rise in extracellular glutamate, excitotoxic injury and progressive paralysis (770). Heterozygote knockouts, on the other hand, show halved concentrations of GLT1, but no apparent morphological brain changes, despite an increased risk of traumatic spinal cord injury (768, 771). Knocking out neuronal GLT1, which represents no more than 10-20% total GLT1 (736, 745), barely affects total GLT1 protein levels and mouse development (772), although it has been implicated in adult neuropsychiatric disorders (773).

Notably, knocking out both major glutamate transporters (GLT1 and GLAST) in mouse, and therefore removing most of the glutamate uptake function, has direct effects on early brain development *in utero* (774). The resulting phenotype involves multiple brain defects (e.g. cortex, hippocampus, and olfactory bulb disorganisation), perinatal mortality (including death *in utero*) and impairment of brain developmental processes (e.g. proliferation of stem cells, differentiation and migration of neurons, survival of subplate neurons). This highlights the indispensability of glutamate transport not only in the postnatal period but also for early brain development. This is key, since abnormal brain development at the foetal stage is increasingly being recognised as a key contributor of many brain disorders that manifest throughout life (55).

1.5.3.2.3.2 Clinical evidence supporting candidacy of glutamate transporter

EAAT2/GLT1 has not been explicitly studied in newborns with HIE. On the other hand, elevated levels have been found in reactive astrocytes and macrophages in and around the necrotic areas of post-mortem brains of very preterm newborns with cPVL (775). This may reflect a response to milder but prolonged hypoxia-ischaemia and/or inflammation, which are harder to disentangle in the preterm brain and may involve reverse transport. Candidacy of EAAT2 is supported by evidence of dysregulation in several neurological, neurodegenerative and psychiatric disorders involving disruption of glutamate signalling, including transient cerebral ischaemia, ischemic stroke, epilepsy, traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, chronic pain, Huntington's disease, HIV-associated dementia, glioma, major depression, schizophrenia, addiction and sleep deprivation (669, 672, 743, 773, 776-792).

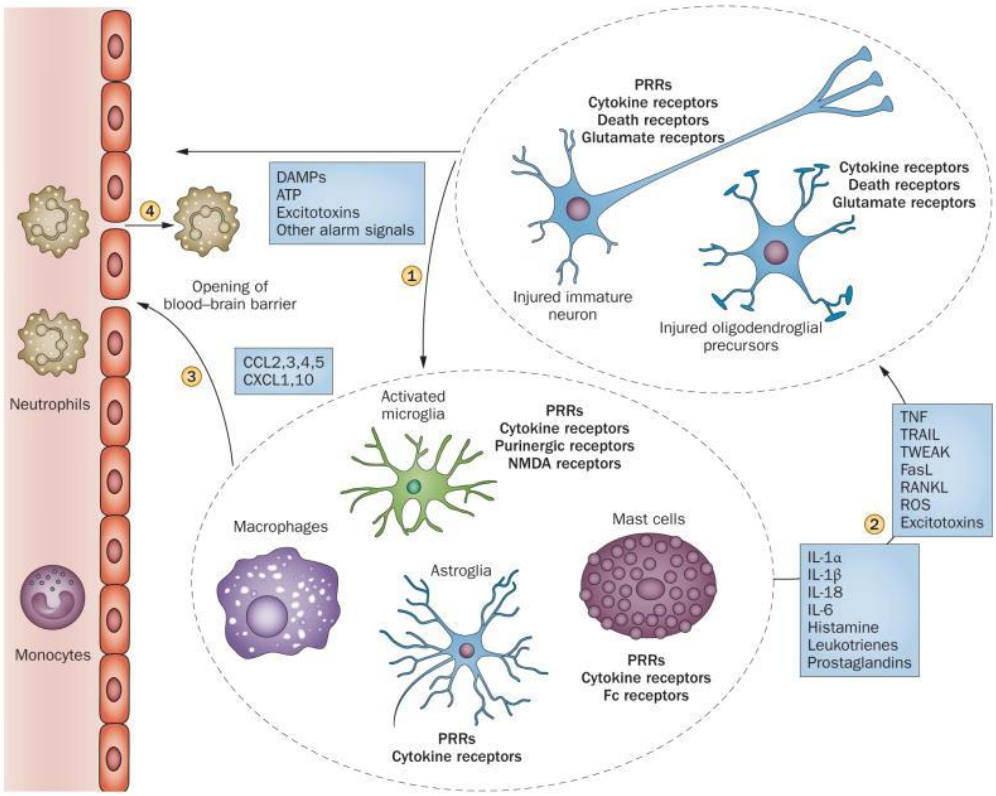
1.5.3.3 Potentiation between neuroinflammation and glutamate excitotoxicity: focus on EAAT2 and TNFα

Preconditioning and postconditioning with mild hypoxia generate ischemic tolerance in the newborn brain (793-798). Similarly, preconditioning with a mild chronic exposure to LPS generates immune tolerance, reducing the magnitude of systemic inflammation (574, 799-802). On the other hand, the mechanisms behind the reciprocal sensitisation between inflammation and HI are still largely unresolved. Additionally, they contrast with the neuroprotective preconditioning effect observed when the interval between a mild inflammatory hit and a subsequent HI hit is intermediate (24h) as opposed to acute (6h) or chronic (72h) in term-equivalent rodents, highlighting that the temporal profile of the insults matters (491, 803). The detrimental sensitising effect of inflammation is thought to be due to

upregulation of pro-inflammatory cytokines and chemokines and activation of apoptotic pathways (491, 803). Another mechanism through which inflammation may sensitise the brain to HI is by increasing the metabolic demands of the brain, though more studies focusing on cerebral oxidative metabolism during inflammation are needed (574, 618, 646).

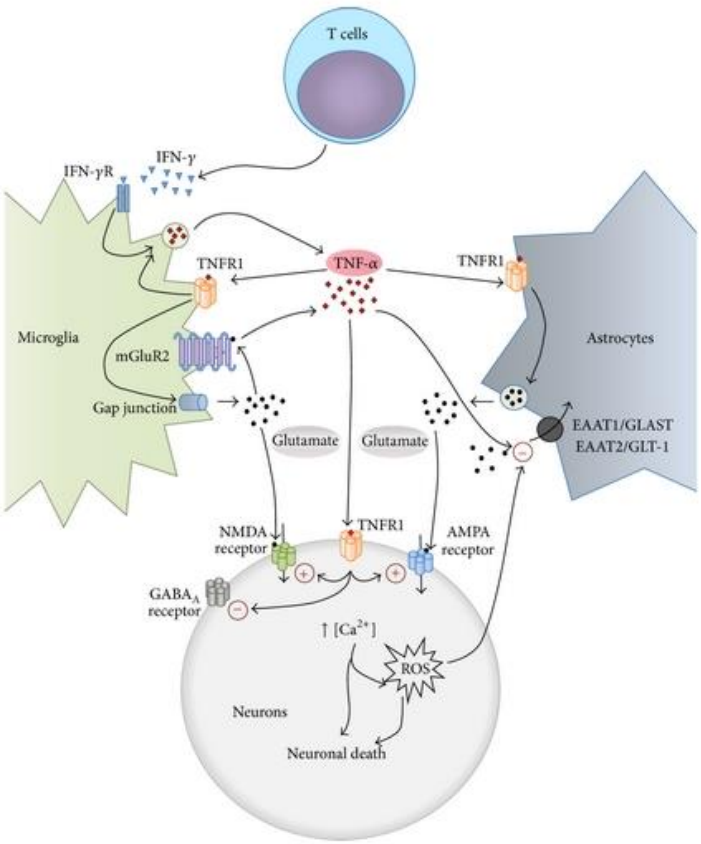
In the current model for sensitisation, HI and neuroinflammation potentiate each other in a bidirectional way, i.e. the excitotoxic injury caused by HI triggers a local inflammatory response in the brain, which in turn further sensitises the brain to HI by exacerbating glutamate excitotoxicity (e.g. impairment of glutamate transport) and further disruption of the blood brain barrier (375, 383, 804). More in detail, neurons and oligodendrocytes injured via excitotoxicity release danger associated molecular patterns (DAMPs) in the microenvironment, which activate microglia inducing cytokine release (1 in Figure 1.8) (375, 805). Microglia can also be activated by peripheral cytokines stimulating endothelial cells to release prostaglandins locally (514, 515), and by cytokines infiltrating from the periphery via the disrupted blood brain barrier and recruiting more peripheral immune cells (494, 513). The net result is a "cytokine storm", whereby the upregulation of pro-inflammatory cytokines is not matched by upregulation of anti-inflammatory cytokines and neurotrophic factors (374, 375, 381, 613, 806-809). The excess pro-inflammatory cytokines further disrupts the blood brain barrier (3 in Figure 1.8), allowing infiltration of more peripheral cytokines and immune cells (4 in Figure 1.8) (375, 544, 810). The excess cytokines and free radicals further damage the developing neurons and oligodendrocytes by enhancing glutamate excitotoxicity (2 in Figure 1.8). Mechanisms are thought to include overactivation of post-synaptic metabotropic glutamate receptors (494, 811, 812), further release of glutamate by activated microglia (813) and failure of the glutamate uptake system, already challenged by the primary energy failure induced by HI.

Figure 1.8. Model for the potentiation between hypoxia-ischaemia and infection/inflammation (375)
1) Cells damaged through excitotoxic injury release endogenous ligands (DAMPs) that generate an inflammatory response. Excess pro-inflammatory cytokines and free radicals further damages 2) neurons and pre-OLCs, via enhancement of glutamate excitotoxicity 3) the vascular bed. This allows 4) infiltration of immune cells from the periphery, which further contribute to the cytokine storm
PPR: pattern recognition receptors of the innate immune system, which recognise both pathogens and DAMPs released by injured cells (375)



One such mechanism of potentiation may involve an interaction between TNFα and EAAT2. TNFα potentiates glutamate excitotoxicity *in vitro* via complex and interacting mechanisms involving crosstalk between neurons, glial cells, and peripheral immune cells, leading to vicious circles of glutamate and cytokine release (Figure 1.9) (544).

Figure 1.9. Molecular mechanisms by which TNFα links glutamate excitotoxicity and inflammation (544)



Infiltrated T cells release IFN-ϒR, which promotes microglial release of TNFα (544).

In pre-oligodendrocytes, TNFα alters glutamate receptor expression and configuration, although AMPA and NMDA blockade is not sufficient to prevent cell death in fetal ovine cell cultures (814).

In neurons, TNFα (via TNFR1) increases glutamate receptor expression and alters their chemical composition making them more permeable to Ca_{2+}, while also decreasing expression of inhibitory $GABA_A$ receptors (544). The net result is an increase in the excitatory strength at the synapsis, which can make neurons more vulnerable to injury in the setting of rising extracellular levels of glutamate and TNFα.

In microglia, two autocrine vicious circles potentiate release of both TNFα and glutamate: firstly, TNFα directly promotes release of more TNFα via TNFR1 signalling (815); secondly, TNFα induces glutamate release by activated microglia by upregulating glutaminase and glutamate release via hemichannels of gap junctions (816). In turn, glutamate activates

glutamate receptors on microglia and induce more TNFα release (817, 818). Dying and injured neurons maintain microglia active.

In astrocytes, TNFα binds to TNFR1, activates COX2 and generates prostaglandin $E_{2,}$ which induces intracellular Ca_{2+} and leads to extracellular glutamate release by astrocytes (814, 819, 820). As well as inducing glutamate release by astrocytes, TNFα impairs astrocytic glutamate transport by selectively suppressing EAAT2 via NFκB (821). Indeed, TNFα causes a dose-dependent suppression of glutamate uptake in human fetal astrocytes *in vitro* sufficient to cause excitotoxicity (821-825), as well as in term-equivalent rat astrocytes during hypoxia (826). Accordingly, inhibiting NFκB is neuroprotective (827). EAAT2 is a double target during hypoxia *in vitro*: not only does hypoxia suppress EAAT1 and EAAT2 directly via disruption of the Na_{+}/K_{+} gradients, but also it promotes TNFα release, which in turn selectively suppresses EAAT2 via NFκB (826). Therefore, it can be hypothesised that other mechanisms leading to upregulation of TNFα (e.g. infection/inflammation, genetically determined high expression) may contribute to suppression of EAAT2 and further exacerbate excitotoxic injury. The relationship between EAAT2 and TNFα appears to be complex, since exposing mouse astrocytes, rat microglia and human blood macrophages to an inflammatory challenge (LPS or TNFα) enhances EAAT2 expression and glutamate uptake function *in vitro* (828-830).

Thus, while the specific mechanisms are largely not known, it is clear that the sensitisation between neuroinflammation and HI-induced glutamate excitotoxicity involves complex interactions between brain cells and a range of mechanisms which result in accumulation of pro-inflammatory cytokines and glutamate in the extracellular space, coupled with loss of normal homeostatic and developmental functions.

1.5.4 Genetic factors

Given the key role of HI and infection/inflammation, it is clear that newborn brain injuries and CP are largely environmental in their causation. However, in the neonatal unit, it is commonly observed that some newborns are more resilient to brain injury than others when exposed to similar insults. Similarly, as many as a third of children diagnosed with CP lack obvious environmental risk factors, while others do particularly well with seemingly complicated clinical histories (831). Not all newborns exposed to perinatal HI and infection/inflammation develop brain injuries and not all newborns with brain abnormalities in the newborn period develop a neurological sequela in childhood. Genetic factors are

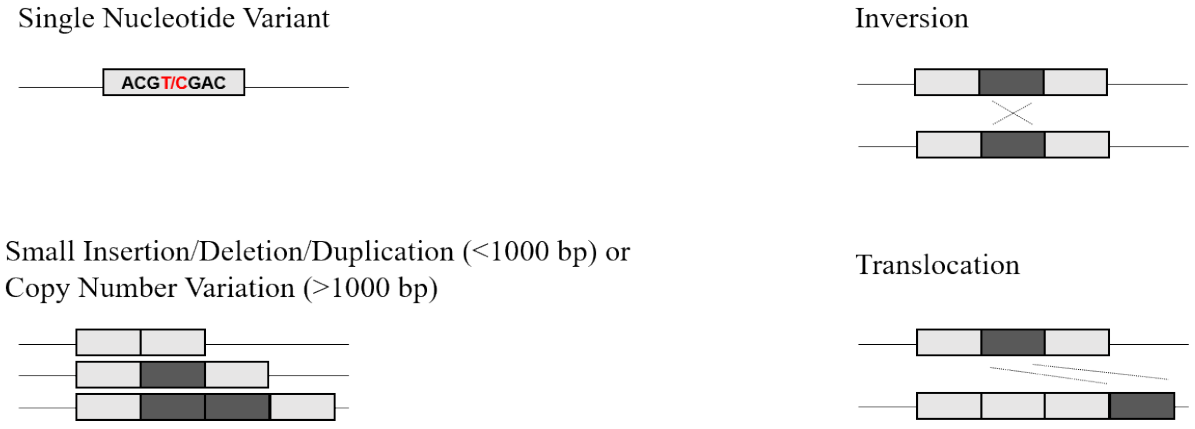
thought to lie at the heart of these discrepancies, with genetic background contributing to the individual response to environmental insults and therefore interindividual differences in vulnerability to injury and impairment. This is analogous to other perinatal disruptions, such as foetal alcohol syndrome, whereby the effects of clear environmental risk factors seem to manifest differentially depending on susceptibility genes (832). Another line of evidence supporting a role for genetic factors in contributing to vulnerability to newborn brain injuries is provided by animal studies, i.e. responses to the same environmental insult (e.g. *in utero* hypoxia) are different between mice and rats (e.g. changes in expression of glutamate-receptor subunits seen in fetal mice but not rats), which are known to have inter-species genetic differences (e.g. extra genes in rats compared to mice, including genes involved in immunity and protein breakdown) (833).

Studies assessing the genetic architecture of neuroimaging outcomes related to brain injuries in the neonatal period are considerably scarcer than classical studies of neurodevelopmental outcomes during childhood. Indeed, enormous progress has been made in the last two decades in our understanding of the genetic basis of neurodevelopmental disorders, including intellectual disability, attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) (834). Studies exploring the genetic factors causing or contributing to CP have also started accumulating (192, 831, 835-841), although they have lagged behind compared to those of other neurodevelopmental disorders, not least because of the difficulty in obtaining large samples. A likely genetic contribution to CP is supported by several lines of evidence. Firstly, the clinical observation that CP often occurs together with neurological and neurodevelopmental comorbidities (e.g. intellectual disability, autism spectrum disorders, epileptic encephalopathies and congenital anomalies) that may overlap not only in the environmental but also in the genetic component, with genetic pleiotropy described for multiple candidate CP genes (837, 842). Early twin and family studies provided evidence of familial aggregation and heritability (843). Some studies have reported a higher CP risk for monozygotic twins (sharing 100% genome) compared to dizygotic twins (sharing 50% genome) (844-846), while others have found higher risk of mortality but no difference in CP rate (847-849). A Swedish population-based study of familial data shows that parents of a child with CP have a 5-fold risk of having a second affected child, with risk reaching 29-fold for twins (850). CP risk is considerably higher in consanguineous families, due to the increased risk of inheriting identical copies of detrimental autosomal recessive genes (851-854). Another line of evidence is provided by studies of parental age, suggesting that older

parental age may increase risk of certain CP subtypes (e.g. dyskinetic cerebral palsy) as the risk of new dominant mutations arises with age (855, 856). Through mathematical modelling in a Swedish study of idiopathic congenital CP, genetic factors were estimated to be likely causes of CP in up to 48% term and 24% preterm cases (857). The advancement in genetic technologies has allowed generation of the first positive evidence of genetic causation in monogenic and copy number variant forms of CP, as well as exploration of the polygenic forms of CP.

Uncovering the genetic architecture of a trait, including cystic and diffuse preterm white matter injury, HIE and CP, means understanding the proportion of variation in each of these traits that is due to genetic factors. This includes identifying all genetic variants affecting disease, their effect size, their frequency in various populations as well as their interactions with each other and the environment (858). This broad-sense heritability can in turn be divided into additive genetic effects (independent and direct effect of each variant, i.e. the narrow-sense heritability), dominance genetic effects (interaction between variants at the same locus) and epistatic genetic effects (interaction between variants at different loci) (859). Genetic variants may be variations at single base pairs in the genome (single nucleotide variants, SNVs), which in turn can be common or rare. Common single nucleotide variants (single nucleotide polymorphisms, SNPs) are present in 5% or more of the population (i.e. the minor allele frequency, or MAF, is ≥ 0.05). Low frequency variants are present in 1-5% of the population and rare variants are present in less than 1% of the population. Genetic variants can also be small (<1,000 bp) insertions or deletions (indels), duplications or inversions of multiple base pairs , as well as large structural changes at the chromosomal level, including copy number variants (CNVs, >1,000 bp) and translocations (860) (see Figure 1.10).

Figure 1.10. Types of genetic variation



1.5.4.1 Rare monogenic forms of cerebral palsy

In monogenic forms of CP, a single mutation is deterministic and responsible for the observed disease outcome. Here, the trait is proximal to the genome, as there are no other contributions other than the genetic variant, which generally results in impaired production of a protein. These mutations are likely to be recent in evolutionary terms, as the large detrimental effects have not yet had time to be selected against (858). Developmental abnormalities deriving from these highly penetrant, large effect variants are more likely to become obvious and be detected earlier in individual patients, often in the absence of MRI evidence of injury (838, 861).

Rare mutations identified in pedigree studies (e.g. AP4M1, GAD1, AP4, ADD3, TUBA1A, SCN8A, KDM5C, NT5C2) often have autosomal recessive mode of inheritance and lead to specific CP subtypes, e.g. quadriplegia with severe intellectual disability, ataxic CP and spastic diplegia (862-869). An imprinted gene mode of inheritance has also been shown in a family with repeated congenital CP (quadriplegia with cognitive impairment), whereby the mutation spans a maternally imprinted gene (KANK1) and is expressed only when inherited from the father (870).

Single-gene forms of CP were initially thought to be rare, representing 1-2% of CP cases (850). However, the most recent estimates from the largest whole-exome sequencing study to date have risen to 14%, with several novel genes being discovered (AGAP1, JHDM1D, MAST1, NAA35, RFX2, WIPI2) (136). At the time of writing, there are 63 genes associated with monogenic forms of CP in the Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org). This suggests that these numbers are likely to increase with increasing sample sizes, favoured by the drop in next-generation sequencing costs and larger collaborations between research study groups. Whole-exome sequencing (WES) allows identification of rare variants in the 1% regions of the genome which code for proteins and are therefore more likely to directly result into a functional protein change (871). Whole-genome sequencing (WGS) can measure nearly all variation in the genome, including large structural variants (see 1.5.4.2), but the high costs have so far limited its uses to research settings. Despite the cost and sample size limitations, sequence data may have immediate clinical applications when causal variants of large effects are identified, in terms of screening, diagnosis, prognosis and therapy. It has been recently suggested that if a genetic cause of CP is discovered via sequencing, the diagnosis of CP should not be changed, since CP remains a

diagnosis based on clinical signs and symptoms, but rather trigger appropriate subclassification based on causation (872).

Interestingly, two *de novo* mutations in the coding regions of EAAT2 have been reported in association with the relevant outcome of early-onset epileptic encephalopathies (873). *De novo* mutations arising for the first time in the egg, sperm, or fertilised egg (i.e. not inherited by either parent) are emerging as more important than expected in neonatal and paediatric neurological diseases, highlighting that causation goes beyond *in utero* disturbances and birth trauma (874).

1.5.4.2 Rare structural variations in cerebral palsy

Copy number variants (CNVs) are rare large structural variations (insertions or deletions) spanning 1000 base pairs (bp) or more, affecting entire chromosomal regions and therefore multiple genes. While studies of CNVs in CP have only recently started to emerge, it is clear that structural variants provide a significant causal contribution to CP. Current estimates suggest that CNVs are responsible for 10-30% CP cases and are likely to be more precisely defined as more studies with larger sample sizes accumulate (192, 875-878). The role of CNVs is better recognised in other neurodevelopmental disorders which have been studied for longer, such as autism, intellectual disability, epilepsy, and schizophrenia (879-882). Indeed, several of the CNVs identified for CP encompass genes that are important for neurological function and are shared with other neurodevelopmental disorders (875, 877). Studies from other neurodevelopmental disorders point to a trend for larger CNVs, which are more likely to arise *de novo*, to be associated with more severe neurodevelopmental impairment (e.g. severe intellectual disability), while smaller inherited variants tend to be associated with less severe impairment (e.g. autism) (883). Accordingly, larger CNVs, including several *de novo* variants, have been found in association with more severe forms of CP based on the Gross Motor Function Classification System (876, 877). This is a very young field and our understanding of the role of CNVs as causes of CP is likely to improve rapidly in the near future, especially as cost of microarray and next generation sequencing technologies continues to drop. CNVs are also emerging in relation to neuropsychiatric disorders in adults born preterm and a prospective neonatal cohort of very preterm newborns is currently being recruited from the National Neonatal Research Database (SPRING cohort) to assess the role of CNVs spanning neurodevelopmental genes (884, 885).

1.5.4.3 Multifactorial polygenic forms of cerebral palsy and common single nucleotide polymorphisms

While some forms of CP are thought to be due to highly penetrant genetic mutations of large effects, other forms of CP are thought to be multifactorial and polygenic. In this different type of genetic architecture, risk can be thought of as an underlying continuous variable, the "disease liability", whereby multiple genetic, developmental, and environmental factors bring a contribution, individually and interactively (886). This includes the collective effect of many common, ancient genetic variants, whose individually small effect is neither sufficient nor necessary, but cumulatively contribute to genetic risk. The disease is manifested when the overall burden of genetic and environmental factors surpasses a threshold of liability (886). Thus, each foetus/newborn will have a different response to adverse environmental risk factors (e.g. perinatal infection/inflammation and hypoxia-ischaemia) due to their underlying genetic background, and therefore a different risk of brain injury and subsequent neurodevelopmental impairment. Polygenic traits encompass many biological processes and causal variants and are therefore distal to the genome (858).

Polygenic traits require large population-based studies to uncover common genetic variants contributing to risk. Genome-wide association studies (GWAS) are currently the standard design to test the direct association (additive, not through interactions) between common genetic variants and a disease outcome (887). In this type of genetic study design, the genome is scanned for variants that are found more frequently in cases than controls, without requiring any *a priori* hypotheses. GWAS have successfully identified many common genetic variants (SNPs) associated with adult disorders, including diabetes, schizophrenia, depression, and bipolar disorder, highlighting the polygenic architecture of many complex traits (888-891). Indeed, the emerging genetic evidence in studies of neurodevelopmental and neuropsychiatric disorders is blurring the lines amongst historically distinct clinical diagnoses and prompting towards a redefinition which takes into account overlapping genetic architectures (892-894). For example, a recent study highlighted a genetic overlap between early motor and sensory development and autism spectrum disorders by school age (895, 896). Given the hypothesis-free design, GWAS require samples in the order of thousands to allow for sufficient power following stringent statistical correction for multiple testing. This has slowed down exploration of childhood neurodevelopmental disorders. While the first significant GWAS findings now exist for autism spectrum disorders and attention deficit

hyperactivity disorder (834, 897, 898), studies of CP have not yet reached sufficiently large samples nationally or internationally.

Candidate-gene studies represent an alternative way to explore potential association based on educated guesses about which genes (or variants in a gene) may be causally involved in disease pathophysiology. These studies have historically preceded hypotheses-free GWAS designs before sufficiently large sample sizes could be achieved. Indeed, several candidate SNP studies have been accumulating in the last 15 years, exploring the association between CP and SNPs affecting selected pathways thought to be central to perinatal brain injuries, namely inflammation, blood coagulation, lipid metabolism, glutamate signalling, nitric oxide metabolism and cell death (162, 163, 654, 836, 840, 899-921). Given the genetic and clinical heterogeneity that characterises CP, a potentially useful approach is to focus on specific populations and pathophysiologies, such as term newborns with HIE or preterm newborns (922-924). The disadvantage of this approach is that it is further penalising in terms of sample size and power. Findings from candidate SNP studies have been inconsistent, highlighting the limitations of current study designs, including small sample sizes, poor replication, clinical and genetic heterogeneity, as well as limited understanding of the underlying disease pathophysiology. Nonetheless, evidence of genetic risk is accumulating and encouraging further exploration with larger sample sizes and integration of different genetic study designs.

Amongst the candidate SNPs explored, regulatory SNPs ultimately affecting regulation of gene expression, and hence the amount rather than the quality of function of the protein, have been amongst these variants. These include common genetic variants in the regulatory regions of cytokines, affecting the magnitude of inflammatory responses to infections, inflammatory comorbidities, and HI (see chapter 2). Despite the evidence of potentiation between inflammation and glutamate excitotoxicity, no studies to date have jointly assessed genetic variants affecting inflammation and glutamate transport in relation to preterm brain injury and neurodevelopmental impairment.

1.5.5 Epigenetic factors

1.5.5.1 Epigenetic mechanisms as mediators of the interaction between genes and environment

Epigenetic mechanisms have been defined as “the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states “ (925). This definition encompasses transient epigenetic marks (e.g. those enabling DNA repair) alongside the more

stable marks, including those inherited from generation to generation. DNA methylation and histone modifications control the transcription of genes from DNA to mRNA by affecting DNA accessibility to the transcriptional machinery. This is achieved via addition or removal of chemical modifications to the DNA sequence or the histone proteins which package DNA into chromatin. Non-coding microRNAs, including different classes of small RNAs (e.g. miRNAs) and long non-coding RNAs (lncRNAs), interact with DNA, mRNA and proteins and provide an additional layer for regulation of expression, for example by controlling transcription, different aspects of mRNA life (e.g. stability, splicing and translation) and chromatin architecture (e.g. direct decompaction, binding of chromatin-remodelling proteins) (926-928). All of these epigenetic mechanisms interact with each other to cooperatively regulate gene expression. Cell function relies on maintenance of epigenetic homeostasis, as highlighted by evidence of epigenomic perturbations in human diseases, such as cancer (929). Within each cell, the epigenome is highly dynamic and is regulated by a complex interplay of genetic, environmental and stochastic factors (930). Genetic background contributes to methylation differences amongst individuals, e.g. SNPs at CpG sites or near CpG sites altering binding of transcription factors or of protein regulating chromatin structure (methylation quantitative trait locus effects, or mQTL) (931, 932). Importantly, epigenetic mechanisms can respond to cues from the environment, including environmental stresses disrupting cellular homeostasis. Such environmentally-induced changes can be propagated from cell to cell within an individual, providing a path to solidification of the gene expression change in the long-term and long after the initial trigger, resulting in maintenance of the phenotype in the organism (933, 934). As well as being propagated from cell to cell within an individual or last for the lifespan in terminally differentiated cells, some epigenetic marks can also be passed onto the next generation without requiring a change in the DNA sequence. For these reasons, epigenetic mechanisms are emerging as the biological mediators linking the effects of genes and environment on cell function, mediating the physical changes induced by the environment on genes and leading to downstream phenotypes in both health and disease (934-937). Effectively, epigenetic mechanisms act as a code of previous experience that results in a differential response to a future exposure (938).

With regard to the vulnerable newborn, this means that: 1) early life stresses, such as the acute hypoxia-ischaemia leading to HIE, may alter the epigenetic landscape in the developing brain and have long-term consequences on brain development and function 2) newborns with the same genetic variants may have different outcomes because of epigenetic differences; 3)

there is a heritable component of risk that is systematically missed out by conventional genetic analyses (939).

1.5.5.2 DNA methylation

Amongst epigenetic mechanisms, DNA methylation has been studied for the longest and benefits from the availability of high-throughput methodologies for profiling (933). It is highly conserved from plants to mammals as a key mechanism for fine spatial and temporal control of gene expression, including during development (940). The observation that promoter DNA methylation correlates with transcriptional repression has led to the notion that cells use this molecular tool to silence gene expression (941). Promoter methylation is thought to inhibit transcription either directly, by inhibiting binding of transcription factors to the promoter, or indirectly, by recruiting methyl-binding proteins that alter chromatin compaction and accessibility to transcription factors (942, 943). Aside from regulation of gene expression, DNA methylation is also key for genomic imprinting of genes, where one parental allele is permanently silenced, inactivation of the X-chromosome, inactivation of transposable elements, and genome stability (944). Relevance to the brain and neurological diseases is highlighted by the fact that, other than the thymus, the brain is the organ with highest levels of DNA methylation (945, 946). Dynamic regulation of DNA methylation is indeed key for brain function across the life span, including neurogenesis, neuronal plasticity, memory formation and maintenance (947-951). Moreover, abnormal DNA methylation patterns are emerging in a range of brain disorders, including autism, schizophrenia and bipolar disorder, stress, anxiety, and addiction (926, 952-961).

DNA methylation represents a suitable candidate mechanism in terms of pathophysiology, due to its ability to alter transcription typically leading to gene silencing, its ability to be propagated from cell to cell and its relative stability over time (933, 962, 963). Despite its general stability, DNA methylation changes occur physiologically in early life and continue to occur during the lifetime in response to environmental stimuli as well as by chance (964, 965). Importantly, epigenetic plasticity and responsiveness to the environment appear to be higher in the earliest and latest stages of life, providing a window of vulnerability in the perinatal period and early childhood (932). Therefore, epigenetic modifications induced by the early life stresses of HI and infection/inflammation may mediate long-term alterations in glutamatergic and inflammatory responses and contribute to long-term neurodevelopmental impairment (966). The relative stability and ease of measurement are also attractive properties for use as a molecular biomarker, while its reversibility introduces the interesting

possibility of pharmacological manipulation for neuroprotection, potentially extending the therapeutic window (743).

DNA methylation involves addition of a methyl group (-CH^3) to the carbon 5 position on the pyrimidine ring of cytosines, resulting in 5-methylcytosines (5-mC) (967). It occurs primarily at cytosines followed by guanines, i.e. CpG sites (cytosine-phosphate-guanine). 60-70% mammalian gene promoters, including those of housekeeping genes, developmental genes, and tissue-specific genes, contain CpG-rich regions of 1-2 kilobases (kb) near the transcription start site (TSS), known as CpG islands. 95% of promoter CpG islands are unmethylated and therefore protected from permanent gene silencing. This is in contrast with the scarce and largely methylated CpG sites scattered throughout the rest of the genome, e.g. in gene bodies (939, 968-973). CpG shores within 2 kb of a CpG island show more variable, dynamic, tissue- and region-specific methylation, and their effect on gene expression is less understood (974-977). Less understood alternative forms of methylation specific to the brain are also emerging, i.e. non-CpG (CpH) methylation and hydroxymethylation (5-hmC) (978-980).

Most of an individual's DNA methylation patterns are established during embryonic and fetal development (939, 981). Gametes carry DNA methylation marks which are largely (though not completely) erased in the zygote immediately after fertilisation, to ensure totipotency for the next generation. Epigenetic marks are subsequently re-established in the primordial germ cells, with partial retention of parental marks (> 40% of all 5-mC), providing a pathway for inheritance of parental DNA methylation in the offspring's sperm and oocytes (982, 983). DNA methylation marks are also re-established in the somatic cells of the zygote after each mitotic DNA replication cycle, where they persist throughout each cell's lifetime ensuring maintenance of cellular identity and function (e.g. neurons and astrocytes from neural progenitor cells) (984, 985). While DNA methylation was originally thought to be stable except for demethylation during early development, it is now clear that it is a dynamic mechanism during development (986).

The "writers" of DNA methylation are DNA methyltransferases (DNMTs), which can establish DNA methylation *de novo* (DNMT3A, DNMT3B, DNMT3L) or identify and maintain existing DNA methylation (DNMT1) (939, 987-991). DNA methylation is subsequently "read" by methyl-CpG-binding proteins (MBDs), such as MECP2, which identify methylated sites and cooperatively recruit histone deacetylases to repress

transcription. Methylation can be erased enzymatically or non-enzymatically. The enzymatic "erasers" of DNA methylation are the ten-eleven translocation (TET) dioxygenases, which actively demethylate DNA by adding a hydroxyl group to 5 methyl cytosine (5-mC) and processing to 5-hydroxymethyl cytosine (5-hmC). While much remains to be uncovered about the functional implications of 5-hmC, we known that 5-hmC is not recognised by DNMTs and methylation status is therefore lost in the daughter cells when DNA is replicated. Demethylation can also occur passively with direct processing to 5-hmC and "methylation dilution" at each DNA replication cycle. Finally, DNA methylation can be inhibited indirectly via destabilisation of the DNMT1 enzyme (939, 984, 992-996).

Global passive DNA demethylation has only been observed during early development in the zygote and primordial germ cells (997). One exception are imprinted genes (e.g. PEG3), which have one parental allele permanently methylated across the lifespan resulting in expression of one parental allele only. Active demethylation has been reported in somatic cells, though only at specific genomic locations and in response to specific stimuli, e.g. active demethylation at BDNF (involved in neural plasticity) following neuronal stimulation, and of the IL2 promoter following T lymphocytes stimulation (997-999). Expression of both the "writers" and "erasers" of DNA methylation has been explicitly shown in during brain development in rodent brain (989, 1000-1002).

1.5.5.3 Perinatal hypoxia-ischaemia and disruption of DNA methylation

Since Barker's hypothesis of the developmental origins of disease, an entire field of research has emerged focusing on the long-term effects of early life stresses on disease (1003-1005). Robust epidemiological studies have generated the hypothesis that adverse environments *in utero* or early childhood can impact later disease outcomes, in a phenomenon known as developmental programming (1006). Landmark studies have linked prenatal exposure to famine (Dutch hunger winter of 1944-45) to risk of schizophrenia and cardiovascular disease (1007-1009). Supported by both epidemiological and experimental studies, epigenetic changes are emerging as the candidate biological mechanisms for such molecular memory (1010-1015).

Perinatal hypoxia is one of the most common early life stresses and the effects of chronic hypoxia during pregnancy on the epigenetic landscape are starting to emerge from experimental studies (985, 1016, 1017). In humans, it is difficult to disentangle the effects of HI from those due to other perinatal exposures in sick newborns. It is also difficult to

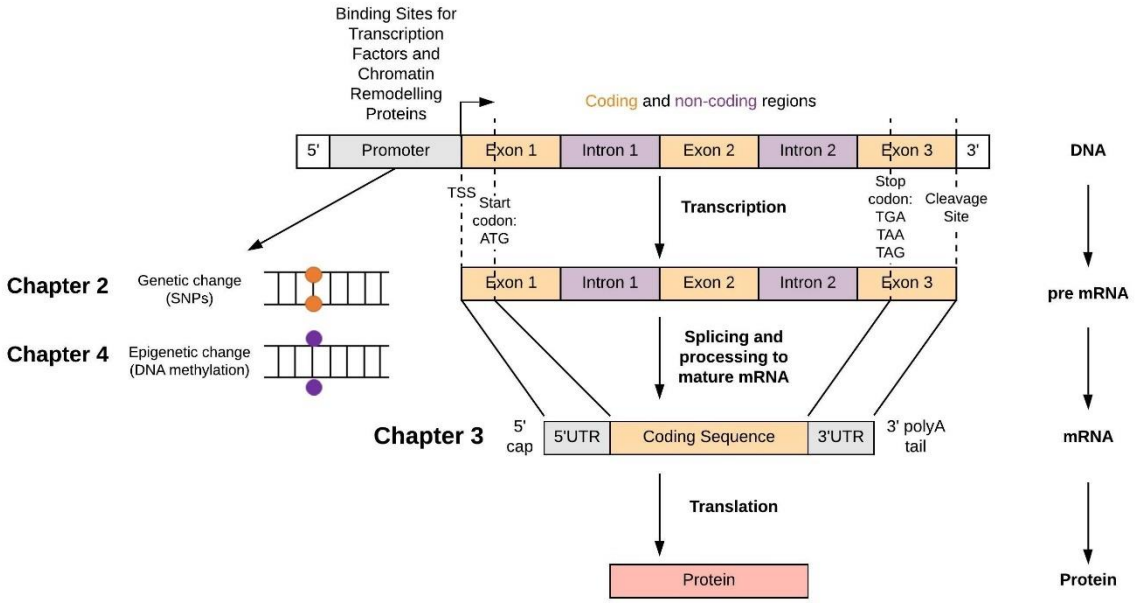
understand which DNA methylation changes are causes or consequences of disease. This is because the temporal sequence between exposure, DNA methylation change and outcome is often not known, and neither is where DNA methylation measurement fits into this temporal sequence (1018). Moreover, assessment of brain methylation is generally not feasible in live newborns and peripheral methylation (e.g. blood) may have limited relevance to the brain (1019). Animal model studies allow evaluation of the causal effect of an insult on the brain's epigenome, providing direct evidence that may translate to humans. These types of studies have shown that epigenetic changes mediate the causal effects of exposure drugs on drug addiction and of maternal care and offspring's stress response (1020, 1021). Crucially, the potential epigenetic consequences of acute peripartum HI leading to HIE have not been explored. This may be achieved by assessing DNA methylation at the candidate glutamatergic and inflammatory genes in the most established rat model of HIE, the Rice Vannucci model (Chapter 3).

1.6 Thesis angle: (dys)regulation of the glutamate transporter and pro-inflammatory cytokines by genetic and epigenetic factors

This project takes a candidate gene approach focusing on the glutamate transporter (*EAAT2*/*Glt1*) and three key pro-inflammatory cytokines (*TNFα*, *IL1β* and *IL6*). The potential role of dysregulation of these genes in perinatal brain injuries and neurodevelopmental impairment is assessed in multiple ways: firstly, by evaluating the effect of regulatory genetic variants on risk of cPVL, motor and cognitive impairment at toddler and pre-school age in a prospective cohort of very preterm newborns (chapter 2); secondly, by evaluating the effects of an acute HI insult on transcription of these genes in an established rat model of HIE at term (chapter 3). Finally, DNA methylation at the promoter of these genes was also assessed as a candidate epigenetic mechanism mediating these transcriptional changes in the brain. Clinical relevance was added by additionally assessing DNA methylation in blood as a potential early biomarker of injury in newborns with HIE (chapter 4). Thus, both genetic and environmental influences on regulation of glutamate transport and inflammation were assessed in relation to two of the most clinically relevant types of newborn brain injuries and subsequent neurodevelopmental impairment. The genetic association study (chapter 2) and the DNA methylation study (chapter 4) are set at the level of the DNA, with the former focusing on changes in the DNA sequence and the latter focusing on chemical modifications laid onto DNA without affecting the sequence. The transcriptional study (chapter 3) assesses

levels of mature mRNA transcripts, which are under both genetic and epigenetic regulation (Figure 1.11).

Figure 1.11. Regulation of gene expression from DNA to protein and context for this project (readapted from (1022))



2 Association between candidate genetic variants affecting glutamate transport and inflammation and neurodevelopmental outcomes in children born preterm

2.1 Introduction

One of the interesting developments from 15 years of genome-wide association studies (GWAS) of complex diseases is that nearly 90% phenotype-associated variants are located in non-coding regions of the genome and are particularly enriched in the regulatory regions, including gene promoters (887, 1023-1025). Regulatory genetic variants influencing gene expression are known as expression quantitative trait loci (eQTL) and underlie genetically programmed differences in mRNA transcription between individuals, via effects on transcription factor binding and epigenetic regulation (1023, 1025).

An approach that can help pinpointing the true causal genetic variants consists in triangulating evidence of:

1) association between altered gene expression and disease, i.e. dysregulated levels of pro-inflammatory cytokines and glutamate transporter in preterm brain injury and neurodevelopmental impairment (see 1.5.3)
2) association between the regulatory SNP and disease, i.e. genetic variants affecting regulation of cytokines and glutamate transporter found at different frequencies in newborns with brain injuries and children with neurodevelopmental impairment
3) association between the regulatory SNP and altered gene expression, i.e. SNP associated with differential gene expression in a relevant cell type, tissue, and developmental stage

With regard to 2), genetic variants in the promoter of three classically studied pro-inflammatory cytokines (*TNFα* -308 G>A, *IL1β* -511 C>T, *IL6* -174 C>G) have been reported in association with poor neurodevelopmental outcomes, including CP, cPVL and neurodevelopmental impairment (see Appendix, Table 8.1 for a literature review) (162, 163, 654, 905, 906, 908, 912, 917-919, 1026-1032). Candidacy is supported by evidence of association with adult autoimmune, infectious, neurological, cardiovascular, and oncological diseases featuring elevated cytokine levels (1033-1040).

SNPs affecting glutamatergic signalling have been historically overlooked. Nonetheless, variants in the glutamate ionotropic NMDA receptor subunits have been reported in

association with motor, cognitive and behavioural outcomes, as well as electrophysiological parameters (921, 1041-1044). Our research group recently reported first evidence of an association between regulatory SNPs in the glutamate transporter (*EAAT2* -200 C>A, -181 A>C) and adverse neurodevelopmental outcomes in very preterm newborns, including CP and low Griffiths/Bayley scores at 2 years (911). Candidacy of the more studied -181 is supported by evidence of association with adverse adult neurological and neuropsychiatric outcomes (e.g. worse neurological outcome after ischemic stroke, bipolar disorder episode recurrence, lower grey matter volumes in bipolar disorder and schizophrenia) (1045-1051).

With regard to 3), SNP functionality has been experimentally demonstrated for the cytokine SNPs via reporter gene assays transfecting mutated promoters in immune cells and assessing promoter activity *in vitro* (1052-1054), and by assessing patients' immune cells *ex vivo* (1055-1059). SNP functionality has been shown also for the *EAAT2* -200 and -181 variants, via a reporter gene assay in fetal rat astrocytes *in vitro* (911), and *in vivo* evidence of -181 association with higher blood glutamate levels in adults with relapsing multiple sclerosis and stroke (1046, 1060).

Given the substantial evidence of potentiation between infection/inflammation and glutamate excitotoxicity (see Chapter 1), it is plausible that newborns with detrimental genetic variants affecting both inflammation and glutamate transport may be particularly vulnerable. In this study, alongside replicating individual SNP-disease outcome associations, we specifically sought to explore the interaction effects (epistasis) between inflammatory SNPs and glutamate transport SNPs on disease risk. This is a secondary analysis using historical genetic and clinical data from the Avon Premature Infant Project (APIP) cohort. This was a Bristol-based prospective cohort of very preterm newborns who participated in a trial assessing the neuroprotective effects of a family-based developmental education programme for parents continued for up to 2 years of life (275, 276). The intervention showed a small positive effect on cognition at 2 years independent of social confounders, which had however attenuated by 5 years.

2.2 Hypothesis

The main hypothesis is that there is an interaction between candidate regulatory SNPs in the glutamate transporter (*EAAT2* -200 and -181) and in three key pro-inflammatory cytokines (*TNFα* -308, *IL1β* -511 and *IL6* -174), so that survivors of very preterm birth (≤32 weeks)

who have risk genotypes at both pathways have a higher risk of CP than those with risk genotypes at either pathway or none.

Additional objectives included:

- Exploring individual SNP-outcomes associations in univariable analyses, including the previously replicated cytokine SNPs and the largely unexplored *EAAT2* SNPs
- Expanding the research question to secondary outcomes including severe white matter injury at birth (cPVL), non-CP motor impairment and cognitive impairment at 2 and 5 years of age

2.3 Materials and methods

2.3.1 Study sample

This is a prospective cohort study using historical genetic and clinical data from the APIP trial (275, 276). This was a cohort of 328 very and extreme preterm newborns (≤32 weeks of gestation) born consecutively in two Bristol hospitals (St Michael's and Southmead Hospitals) between 1990 and 1993. Children with major congenital anomalies of the central nervous system and genetic syndromes known to cause CP or neurodevelopmental impairment were excluded from the original trial. All families spoke English as the first language at home. Of the original 328 APIP newborns, the current analysis excluded the 20 newborns who died before discharge and included the 308 survivors.

2.3.2 Genetic variants

Genetic data originated from routine blood spot screening card collected within the first 5-8 days after birth as part of the UK Newborn Screening Programme (http://newbornbloodspot.screening.nhs.uk). The 5 candidate SNPs were genotyped in the APIP cohort in 2004 (inflammatory SNPs) and 2014 (glutamatergic SNPs) (Table 2.1). Genotypes for the 308 survivors were obtained directly from the authors in text format.

Table 2.1. List of candidate SNPs

Gene	Chromosome	SNP			Reference
		Genomic Position from TSS	*SNP Type*	*SNP ID*	
EAAT2	11	g.-200 g.-181	C>A A>C	rs111885243 rs4354668	(911, 1061)
TNFα	6	g.-308	G>A	rs1800629	Harding, D (unpublished)
IL1β	2	g.-511	C>T	rs16944	Harding, D (unpublished)
IL6	7	g.-174	C>G	rs1800795	(1030)

Genetic data was obtained by the original authors using different DNA extraction and genotyping methodologies since the studies were conducted by separate research groups a decade apart. For the cytokines, DNA was extracted by boiling blood spots in sterile water followed by phenol extraction. The regions of interest in the cytokine promoters were amplified by polymerase chain reaction (PCR) using commercially available primers and the PCR products digested with restriction enzyme NLaIII (Restriction Fragment Length Polymorphism analysis, RFLP analysis). Resulting amplified products are cut or uncut depending on the presence of the allele and can be separated by size. This was achieved using microtiter array diagonal gel electrophoresis (MADGE), which allows to run multiple samples simultaneously in a 96-well plate.

For the glutamate transporter, DNA was extracted from blood spots using a commercial kit with extraction columns (QIAamp DNA Micro Kit, Qiagen) (1062). The region of interest in the promoter was amplified by PCR using custom primers, including a biotinylated forward primer. After denaturation of amplified DNA, the single strands tagged with biotin were selectively bound to streptavidin-coated beads, ready for pyrosequencing. This technique generates a light signal every time a nucleotide complementary to the target sequence is incorporated, allowing sequencing by synthesis in real-time.

Due to their high but incomplete linkage disequilibrium (LD), the *EAAT2* SNPs were additionally coded as nine -200/-181 genotype combinations, as previously described (911). Moreover, a new exposure variable was created for the number of A alleles at the -200/-181 genotype combination, based on previous evidence of association with adverse neurodevelopmental outcomes in a larger sample including APIP (911).

Linkage disequilibrium between all genetic variants was measured in the complete cases cohort using the Haploview 4.2 software (https://www.broadinstitute.org/haploview/). A linkage and a marker file were created in tab-delimited text format (https://www.broadinstitute.org/haploview/input-file-formats). The linkage file contained the newborns' IDs and genotypes, the marker file contained the SNPs IDs and genomic locations. Both files were uploaded on Haploview, deselecting the default settings of removing individuals with <50% genotypes and SNPs >500 kb apart. LD was reported with both D' (ranging -1 to 1) and r^2 (ranging 0 to 1) coefficients. Both coefficients measure the amount of LD in a sample from the population. The consideration for both D' and r^2 allows to discriminate between perfect LD (D'=1; r^2=1) and complete LD (D'=1; r^2<1).

2.3.3 Clinical outcomes

The primary outcome measure was a diagnosis of CP at 2 years, assigned by a clinical academic paediatrician or a research physiotherapist using standardised definitions (1063).

Secondary outcomes were:

1) cPVL, diagnosed by cranial ultrasound by a clinical academic neonatologist during the neonatal stay and defined using standard guidelines (i.e. echo-lucent cysts of ≥2 mm diameter in the periventricular white matter) (4)
2) Griffiths aggregate general quotient (AGQ), assessing neurodevelopment at 2 years and administered by a psychologist (1064). The AGQ is derived from five subscales: personal and social, hearing and speech, locomotor, eye hand co-ordination and performance domains
3) British Ability Scales score (BAS, 2nd Ed.), assessing cognitive skills at 5 years and administered by a psychologist (1065). The general conceptual ability score (GCA), an IQ estimate, is derived from verbal, non-verbal reasoning, and spatial skills subscales.

   Both the Griffiths DQ and BAS GCA have a standardised mean of 100 (± 15 SD) in the UK reference population, with lower scores indicating lower performance (Harding et al., 2004, Harding et al., 2005)
4) Movement ABC Score (M-ABC, 1st Ed.), assessing manual dexterity, coordination and balance at 5 years and administered by a research nurse (216, 1066). The impairment score normalised to the reference UK population ranges from 0 to 40: 0-

10 is classified as normal (>15th percentile), 10.5-16.5 as borderline (15th-5th percentile), 17-40 as definite movement impairment (<5th percentile)

Data on intraventricular haemorrhage was also available, with grading following the Papile classification (1067). Sensitivity analyses included additional measures of moderate white matter injury: persistent periventricular echodensities (echodensities present after 14 days of life not developing into cysts) and ventricular dilatation (ventricular size >97th percentile) (1068).

2.3.4 Clinical covariates

Birth weight, gestational age and biological sex were considered *a priori* as possible mediators/effect modifiers and were used to generate z-scores standardised to the UK-WHO Preterm Growth Reference (1069). Newborns below the 10th birth centile (z-score ≤-1.28) were considered *a priori* as small for gestational age. Multiple births and a range of perinatal inflammatory exposures were also considered *a priori* as possible mediators/effect modifiers, including maternal infection/chorioamnionitis, early and late neonatal sepsis and necrotising enterocolitis. Ethnicity information was available only in the form of Caucasian/non-Caucasian, with all ethnic minority groups grouped together.

2.3.5 Statistical analysis

The complete cases cohort (n=202) was defined as the subset of newborns with genotype data for both glutamate transporter SNPs and at least one cytokine SNP, as well as primary CP outcome data. Analyses involving each variant independently or each *EAAT2*-cytokine pair had different complete cases subsets, and, as such, denominators vary throughout the study.

Allele frequencies in the complete cases cohort (n=202) were compared to those in the original APIP cohort (n=308) with a two-sample test of proportions. They were also compared to allele frequencies in the European population of the 1000 Genomes Project (see 2.3.6). Hardy-Weinberg disequilibrium (HWD) was tested for all SNPs with a χ^2 test and a threshold of $p<0.05$.

Associations with neurodevelopmental outcomes were assessed for each variant independently with univariable tests of associations. Categorical outcomes were analysed by χ^2 or Fisher's exact test (if cell n<5). In *post hoc* analyses, genotypes with significantly different frequencies were identified as those with adjusted Pearson residuals >1.96 (i.e. more than two standard deviations from the mean). Continuous outcomes were analysed with one-way ANOVA for variables with expected normal distribution and Kruskal Wallis test for

variables with expected skewed distribution. In *post hoc* analyses, the Dunnett's test was used to compare the mean outcome for each of the two risk genotypes to that of the reference genotype. For categorical outcomes, effect sizes were estimated as odds ratios relative to the reference genotype (OR_{aa} and OR_{Aa}). For continuous outcomes, effect sizes were expressed as mean differences ($\bar{x}_{AA}$-$\bar{x}_{aa}$; $\bar{x}_{AA}$-$\bar{x}_{Aa}$).

Following univariable analysis, multivariable regression analysis was planned to explore the interaction between the number of A alleles at *EAAT2* -200/-181 (as a continuous variable) and each cytokine variant (as a categorical variable with three levels/genotypes), as well as to explore the effect of clinical covariates. Goodness of fit of increasingly complex models was tested and compared with the likelihood ratio test: baseline model vs univariate model, univariate model vs bivariate model (main effects), bivariate model (main effects) vs bivariate model (interaction). For the M-ABC score, which is positively skewed by definition, two approaches were considered: a linear regression with log transformed scores, or, in case of large departures from linear assumptions, a generalised linear model approach (gamma family, log link).

Bonferroni correction was not applied in this exploratory hypothesis-driven study; however, all comparisons are reported. All analyses were performed using Stata 14 (Stata Corp, TX, USA).

2.3.6 Comparison with 1000 Genomes Project

To compare our data to that of a large reference sample, allele frequency and Hardy-Weinberg data were obtained from the 1000 Genomes Project (http://www.internationalgenome.org). Allele frequency data was obtained from the most recent build (dbSNP build 153) of the dbSNP database of short genetic variations from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/snp/).

Hardy-Weinberg data was extracted from the latest release 1000 Genomes Phase 3 (20130502 release) for the total sample, as well as the British subset and the CEU subset (Utah Residents with Northern and Western European Ancestry), which are both part of the larger European subset. Publicly available genotype data were downloaded from the FTP website (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/) in Variant Call Format (VCF) using the Data Slicer web tool (https://www.internationalgenome.org/data-slicer/) to filter and extract data by population. The downloaded VCF files were converted to suitable PED file format,

ready for Hardy-Weinberg equilibrium (HWE) data extraction with the Plink 2.0 software (www.cog-genomics.org/plink/2.0/).

2.4 Results

2.4.1 Complete cases

The complete cases cohort with data for both *EAAT2* SNPs, at least one cytokine and CP outcome included 202 newborns. Complete cases cohorts were smaller for Griffiths (n=178), M-ABC (n=137) and BAS score (n=129). Individual complete cases cohorts for each *EAAT2*-cytokine SNP pair included between 31% and 58% of the original APIP cohort (Table 2.2).

Table 2.2. Complete cases cohorts for each *EAAT2* SNPs-cytokine SNP pair

Complete Cases Cohorts	**Clinical Outcomes**				
	CP	*cPVL*	*Griffiths*	*BAS*	*M-ABC*
TNFα -308	178 (58%)	175 (57%)	156 (51%)	114 (37%)	121 (39%)
IL1β -511	170 (55%)	167 (54%)	152 (49%)	109 (35%)	118 (38%)
IL6 -174	140 (45%)	137 (44%)	124 (40%)	89 (29%)	96 (31%)

Numbers are counts of children with genetic data for both *EAAT2* SNPs, each cytokine SNP and relevant outcome

2.4.2 Distribution of alleles and genotypes

Genotype distributions of the five candidate SNPs are shown in Table 2.3.

Table 2.3. Genotype distributions

	***EAAT2* -200**	***EAAT2* -181**	***TNFα* -308**	***IL1β* -511**	***IL6* -174**
Genotype Frequency	CC	AA	GG	CC	GG
	50 (24.8%)	45 (22.3%)	100 (56.2%)	84 (49.4%)	25 (17.9%)
	CA	AC	GA	CT	GC
	116 (57.4%)	116 (57.4%)	68 (38.2%)	66 (38.8%)	67 (47.9%)
	AA	CC	AA	TT	CC
	36 (17.8%)	41 (20.3%)	10 (5.6%)	20 (11.8%)	48 (34.3%)
Total n	202	202	178	170	140

Allele frequencies in the complete cases cohort were not significantly different from those in the original APIP cohort (Table 2.4). The *TNFα* -308 minor A allele was significantly more frequent in our cohort compared to the 1000 Genomes Project European subset (p<0.001). There was also some evidence that the *EAAT2* variants were more frequent in our cohort (-181: p=0.006; -200: p=0.006).

Table 2.4. Allele frequencies

Genetic Variant	**Allele Frequencies**							
	Complete Cases Cohort		*Vs Original APIP Cohort*			*Vs 1000 Genomes (EUR)*		
		n		*n*	*p**		*n*	*p**
EAAT2 -200	C: 0.53	404	C: 0.54	408	0.775	C: 0.61	1006	**0.006**
	A: 0.47		A: 0.46			A: 0.39		
EAAT2 -181	A: 0.51	404	A: 0.51	408	>0.999	A: 0.59	1006	**0.006**
	C: 0.49		C: 0.49			C: 0.42		
TNFα -308	G: 0.75	356	G: 0.77	414	0.516	G: 0.87	1006	**<0.001**
	A: 0.25		A: 0.23			A: 0.13		
IL1β -511	C: 0.69	340	C: 0.68	402	0.770	C: 0.65	1006	0.178
	T: 0.31		T: 0.32			T: 0.35		
IL6 -174	C: 0.42	280	C: 0.43	328	0.804	C: 0.42	1006	>0.999
	G: 0.58		G: 0.57			G: 0.58		

*Two-sample test of proportions vs complete cases cohort

Hardy-Weinberg equilibrium was tested for all SNPs. There was some evidence that the *EAAT2* SNPs were in Hardy-Weinberg disequilibrium (-181: p=0.034; -200: p=0.029), i.e. the observed genotype frequencies were significantly different to the genotype frequencies expected from the observed allele frequencies under the Hardy-Weinberg assumptions. Specifically, excess heterozygotes were observed for both variants (Table 2.5). The *EAAT2* variants were also found to be in Hardy-Weinberg disequilibrium in the total 1000 Genomes Phase 3 sample, which includes genomic data for 2,504 individuals, as well as the British subset (n=91). However, in these two samples from the reference population, the direction was opposite with loss of heterozygotes.

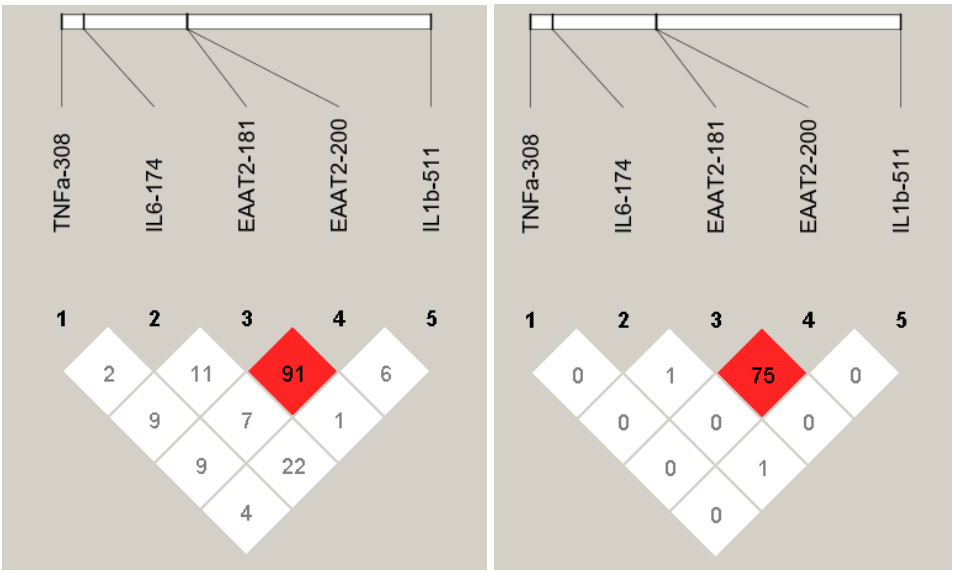
Table 2.5. Hardy-Weinberg disequilibrium testing for the *EAAT2* SNPs

Population	n	*EAAT2* -181		*EAAT2* -200	
		p	Heterozygote Frequencies	p	Heterozygote Frequencies
Current Study Sample	202	**0.034**	Observed: 0.57 Expected: 0.50	**0.029**	Observed: 0.57 Expected: 0.50
GBR (British in England and Scotland)	91	**0.05**	Observed: 0.38 Expected: 0.48	0.09	Observed: 0.40 Expected: 0.48
CEU (Utah Residents with Northern and Western European Ancestry)	99	0.533	Observed: 0.51 Expected: 0.48	0.67	Observed: 0.49 Expected: 0.47
Total 1000 Phase 3 Sample	2504	**$5.16*10^{-15}$**	Observed: 0.40 Expected: 0.47	**$7.43*10^{-14}$**	Observed: 0.39 Expected: 0.46

While the cytokine SNPs show very little non-random association ($r^2 = 0.01$, D' < 0.2), the *EAAT2* SNPs were confirmed to be in strong but incomplete linkage disequilibrium ($r^2 = 0.8$, D' = 0.9).

Figure 2.1. Linkage disequilibrium plots from Haploview. D' (left) and r^2 (right)

The SNPs appear to be on the same chromosome only because of the software output



The distributions of the 9 genotype combinations of *EAAT2* -200 and -181 are shown in Table 2.6. 89% (n=180) children had concordant genotype combinations with 2 A alleles, i.e.

if -200 was homozygous (CC or AA), then -181 was also homozygous (AA or CC) and if -200 was heterozygous (CA or AC) then -181 was also heterozygous (AC or CA). Over half of these children were heterozygote at both SNPs (genotype combination 2), followed by homozygote for major allele at both SNPs (genotype combination 1) and homozygote for the minor allele at both SNPs (genotype combination 3). Only 11% children had discordant genotype combinations, i.e. if -200 was homozygous then -181 was heterozygous or vice versa. Of these, 8% (n=16) of the children had 1 A allele (genotype combinations 6 and 9) and 3% (n=6) had 3 A alleles (genotype combinations 4 and 7). There were no children with the rarest genotype combinations 5 and 8 in this cohort (i.e. 4 As and 4 Cs).

Table 2.6. Distribution of the *EAAT2* -200/-181 genotype combinations

No. A alleles at -200/-181	*EAAT2* -200	*EAAT2* -181	Frequency	
0	CC	CC	0	-
1	CC	AC	9 (4.5%)	16 (8%)
	CA	CC	7 (3.5%)	
2	CC	AA	41 (20.3%)	180 (89%)
	CA	AC	105 (52.0%)	
	AA	CC	34 (16.8%)	
3	CA	AA	4 (2.0%)	6 (3%)
	AA	AC	2 (1.0%)	
4	AA	AA	0	-
Total			202	

2.4.3 Characteristics of the cohort

Clinical and demographic characteristics of the complete cases cohort are shown in Table 2.7. There was no evidence that the children not included in the analyses due to missing genetic and/or CP data had different demographic/clinical characteristics compared to those in the complete cases cohort.

Table 2.7. Clinical and demographic characteristics of the cohort

Perinatal Characteristics	Complete Cases Cohort		Missing Subset		p
	n		*n*		
Male Sex	202	114 (56.4%)	106	63 (59.4%)	0.613
Birth Weight (g)	202	1432.4 (±387.9)	105	1444.5 (±391.3)	0.797
Gestational Age (weeks)	202	30 (29-32)	105	31 (29-32)	0.694
Small for Gestational Age	201	19 (9.5%)	105	11 (10.5%)	0.775
Multiple Birth	202	41 (20.3%)	106	17 (16.0%)	0.364
Maternal Infection	200	13 (6.5%)	104	6 (5.8%)	0.803
Neonatal Sepsis	202	69 (34.2%)	105	32 (30.5%)	0.515
Necrotising Enterocolitis	199	8 (4.0%)	101	4 (4.00%)	0.980
Caucasian Ethnicity	201	184 (91.5%)	104	95 (91.4%)	0.954

Within the complete cases cohort, 86% (n=173) of the children were born very preterm (28-32 weeks) and 14% (n=29) extreme preterm (<28 weeks) (Table 2.8).

Table 2.8. Distribution of gestational ages

Gestational Age (weeks)	n
22	1 (0.5%)
23	-
24	1 (0.5%)
25	7 (3.5%)
26	8 (4.0%)
27	12 (5.9%)
28	15 (7.4%)
29	29 (14.4%)
30	32 (15.8%)
31	43 (21.3%)
32	54 (26.7%)
n (tot)	202

Clinical and demographic characteristics of the complete cases cohort split by genotype are shown in Table 2.9, Table 2.10, Table 2.11, Table 2.12, Table 2.13 and Table 2.14. Ethnicity was strongly associated with the number of A alleles at *EAAT2* -200/-181 (Table 2.11). In the *post hoc* analysis, it was found that, while the two *EAAT2* SNPs did not differ by ethnicity in terms of minor allele frequency (-200, p>0.999; -181, p=0.373), non-Caucasian children were more likely to have discordant genotypes than Caucasian children (41.2% vs 8.2%, p<0.001).

There was some evidence that *IL1β* -511 was associated with being small for gestational age (p=0.041) (Table 2.13), with more SGA in the reference CC genotype (adjusted Pearson residual = 2.57). There was very weak evidence of the following associations: *EAAT2* -200 and birth weight (p=0.068) (Table 2.9), *TNFα* -308 and gestational age (p=0.081) (Table 2.12), and *IL6* -174 and ethnicity (p=0.078) (Table 2.14).

Table 2.9. Clinical and demographic characteristics by *EAAT2* -200 genotype

Perinatal Characteristics	***EAAT2* -200**							**p**
		CC		*CA*		*AA*		
	Total		*n*		*n*		*n*	
Male sex	202	28 (56.0%)	50	60 (51.7%)	116	26 (72.2%)	36	0.095
Birth weight (g)	202	1452.7 (±400.4)	50	1385.8 (±363.1)	116	1554.3 (±428.5)	36	0.068
Gestational Age (wk)	202	31 (28-32)	50	30 (29-32)	116	31 (29.5-32)	36	0.354
Small for Gestational Age	201	4 (8.0%)	50	14 (12.1%)	116	1 (2.9%)	35	0.282
Multiple Birth	202	9 (18.0%)	50	24 (20.7%)	116	8 (22.2%)	36	0.880
Maternal Infection	200	3 (6.1%)	49	7 (6.0%)	116	3 (8.6%)	35	0.855
Neonatal Sepsis	202	16 (32.0%)	50	40 (34.5%)	116	13 (36.1%)	36	0.918
Necrotising Enterocolitis	199	0 (0.0%)	49	6 (5.2%)	115	2 (5.7%)	35	0.209
Caucasian ethnicity	201	45 (91.8%)	49	106 (91.4%)	116	33 (91.7%)	36	0.995

Table 2.10. Clinical and demographic characteristics by *EAAT2* -181 genotype

Perinatal Characteristics		*EAAT2* -181						p
		CC		*CA*		*AA*		
	Total		*n*		*n*		*n*	
Male sex	202	27 (60.0%)	45	60 (51.7%)	116	27 (65.9%)	41	0.252
Birth weight (g)	202	1470.0 (±391.4)	45	1388.8 (±366.4)	116	1514.5 (±433.4)	41	0.156
Gestational Age (wk)	202	31 (28-32)	45	30 (29-31)	116	31 (29-32)	41	0.236
Small for Gestational Age	201	3 (6.7%)	45	15 (12.9%)	116	1 (2.5%)	40	0.140
Multiple Birth	202	6 (13.3%)	45	28 (24.1%)	116	7 (17.1%)	41	0.263
Maternal Infection	200	3 (6.7%)	45	7 (6.1%)	114	3 (7.3%)	41	0.928
Neonatal Sepsis	202	15 (33.3%)	45	39 (36.2%)	116	15 (36.6%)	41	0.934
Necrotising Enterocolitis	199	0 (0.0%)	45	6 (5.3%)	114	2 (5.0%)	40	0.293
Caucasian ethnicity	201	42 (95.5%)	44	107 (92.2%)	116	35 (85.4%)	41	0.227

Table 2.11. Clinical and demographic characteristics by number of A alleles at *EAAT2* -200/-181

Perinatal Characteristics	No. A alleles at *EAAT2* -200/-181							p
		1		*2*		*3*		
	Total		*n*		*n*		*n*	
Male sex	202	6 (37.5%)	16	104 (57.8%)	180	4 (66.7%)	6	0.256
Birth weight (g)	202	1373.9 (±366.8)	16	1433.4 (±395.5)	180	1559.3 (±124.2)	6	0.607
Gestational Age (wk)	202	31 (29-31)	16	30 (29-32)	180	31 (30-32)	6	0.459
Small for Gestational Age	201	1 (6.3%)	16	18 (10.1%)	179	0 (0.0%)	6	>0.999
Multiple Birth	202	4 (25.0%)	16	35 (19.4%)	180	2 (33.3%)	6	0.497
Maternal Infection	200	0 (0.0%)	15	13 (7.2%)	180	0 (0.0%)	5	0.718
Neonatal Sepsis	202	4 (25.0%)	16	64 (35.6%)	180	1 (16.7%)	6	0.556
Necrotising Enterocolitis	199	0 (0.0%)	15	8 (4.5%)	178	0 (0.0%)	6	>0.999
Caucasian ethnicity	201	10 (62.5%)	16	169 (94.4%)	179	5 (83.3%)	6	**<0.001**

Table 2.12. Clinical and demographic characteristics by *TNFα* -308 genotype

Perinatal Characteristics		*TNFα* -308						p
		GG		*GA*		*AA*		
	Total		*n*		*n*		*n*	
Male sex	178	59 (59.0%)	100	35 (51.5%)	68	7 (70.0%)	10	0.429
Birth weight (g)	178	1,447.9 (±410.4)	100	1,426.3 (±367.1)	68	1,269.9 (±357.1)	10	0.391
Gestational Age (wk)	178	30 (28-32)	100	30 (29-31)	68	29 (26-30)	10	0.081
Small for Gestational Age	177	7 (7.0%)	100	8 (11.9%)	67	0 (0.0%)	10	0.418
Multiple Birth	178	22 (22.0%)	100	15 (22.1%)	68	2 (20.0%)	10	>0.999
Maternal Infection	177	8 (8.0%)	100	3 (4.5%)	67	1 (10.0%)	10	0.447
Neonatal Sepsis	178	37 (37.0%)	100	23 (33.8%)	68	5 (50.0%)	10	0.622
Necrotising Enterocolitis	175	2 (2.0%)	98	4 (6.0%)	67	1 (10.0%)	10	0.146
Caucasian ethnicity	177	90 (90.9%)	99	63 (92.7%)	68	9 (90.0%)	10	0.910

Table 2.13. Clinical and demographic characteristics by *IL1β* -511 genotype

Perinatal Characteristics		***IL1β* -511**						**p**
		CC		*CT*		*TT*		
	Total		*n*		*n*		*n*	
Male sex	170	44 (52.4%)	84	41 (62.1%)	66	10 (50.0%)	20	0.419
Birth weight (g)	170	1430 (±386.8)	84	1432.1 (±402.1)	66	1443.9 (±392.8)	20	0.999
Gestational Age (wk)	170	30 (29-32)	84	30 (29-32)	66	31 (28.5-32)	20	0.899
Small for Gestational Age	169	14 (16.9%)	83	3 (4.6%)	66	1 (5.0%)	20	**0.041**
Multiple Birth	170	15 (17.9%)	84	15 (22.7%)	66	4 (20.0%)	20	0.738
Maternal Infection	168	8 (9.6%)	83	1 (1.5%)	65	2 (10.0%)	20	0.092
Neonatal Sepsis	170	31 (36.9%)	84	21 (31.8%)	66	6 (30.0%)	20	0.742
Necrotising Enterocolitis	167	4 (4.8%)	83	2 (3.1%)	65	0 (0.0%)	19	0.854
Caucasian ethnicity	169	77 (91.7%)	84	58 (89.2%)	65	18 (90.0%)	20	0.878

Table 2.14. Clinical and demographic characteristics by *IL6* -174 genotype

Perinatal Characteristics	*IL6* -174							p
		GG		*GC*		*CC*		
	Total		*n*		*n*		*n*	
Male sex	140	31 (64.6%)	48	41 (61.2%)	67	12 (48.0%)	25	0.375
Birth weight (g)	140	1457.4 (±393.6)	48	1440.4 (±442.7)	67	1468.7 (±287.2)	25	0.948
Gestational Age (wk)	140	30 (29-31)	48	31 (29-32)	67	30 (29-31)	25	0.906
Small for Gestational Age	139	3 (6.3%)	48	7 (10.6%)	66	2 (8.0%)	25	0.852
Multiple Birth	140	8 (16.7%)	48	13 (19.4%)	67	6 (24.0%)	25	0.752
Maternal Infection	139	1 (2.1%)	48	8 (12.1%)	66	1 (4.0%)	25	0.103
Neonatal Sepsis	140	22 (45.8%)	48	21 (31.3%)	67	7 (28.0%)	25	0.188
Necrotising Enterocolitis	139	3 (6.4%)	47	4 (6.0%)	67	0 (0.0%)	25	0.674
Caucasian ethnicity	139	41 (85.4%)	48	64 (97.0%)	66	23 (92.0%)	25	0.078

2.4.4 Outcomes

Outcome distributions are shown in Table 2.15. There was no evidence that the children not included in the analyses due to missing genetic and/or CP data had different outcomes compared to those in the complete cases cohort. Within the complete cases cohort, children with missing data for the BAS score at 5 years were more likely to have had a CP diagnosis at 2 years (p=0.042). Similarly, children with missing M-ABC assessment at 5 years were more likely to have had a CP diagnosis (p=0.003). There was no significant evidence of an association between the Griffith score at 2 years and a missing BAS score (p=0.179) or M-ABC score (p=0.273) at 5 years.

Table 2.15. Outcome distributions

Outcome	**Complete Cases Cohort**		**Missing Subset**		**p**
	n		*n*		
Cerebral Palsy	202	17 (8.4%)	106	13 (12.3%)	0.279
Diplegia		8 (4.0%)		1 (0.9%)	
Quadriplegia		8 (4.0%)		4 (3.8%)	
Hemiplegia		1 (0.5%)		2 (1.9%)	
Hypotonia		-		4 (3.8%)	
Mixed		-		2 (1.9%)	
cPVL	199	15 (7.5%)	95	8 (8.4%)	0.792
Griffiths	178	96.0 (±15.1)	89	93.73 (±19.5)	0.306
BAS	129	98.3 (±15.7)	68	101.82 (±16.3)	0.137
M-ABC	137	5 (2-12)	72	4.75 (1.75-9.5)	0.715

Summary results of the univariable tests of associations are reported in Table 2.16 for the glutamate transporter variants and Table 2.17 for the cytokines variants. The results will also be presented by outcome in the relevant paragraphs.

Table 2.16. Univariable tests of association between the *EAAT2* SNPs and all outcomes

Outcomes	***EAAT2* -200**							
		CC		*CA*		*AA*		*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	202	4 (8.0%)	50	10 (8.6%)	116	3 (8.3%)	36	>0.999
cPVL	199	3 (6.0%)	50	10 (8.8%)	114	2 (5.7%)	35	0.812
Griffith	178	96.7 (± 16.6)	46	95.7 (± 16.0)	99	95.7 (± 9.7)	33	0.936
BAS	129	99 (± 15)	34	100 (± 15)	71	92 (± 18)	24	0.084
M-ABC	137	3.5 (0.5-10)	35	5 (2 – 11.5)	81	7 (3 – 12.5)	21	0.440
	***EAAT2* -181**							
		AA		*AC*		*CC*		*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	202	5 (11.1%)	45	10 (8.6%)	116	2 (4.9%)	41	0.690
cPVL	199	3 (6.7%)	45	9 (8.0%)	113	3 (7.3%)	41	>0.999
Griffith	178	95.6 (±18.3)	42	96.2 (±15.5)	98	95.9 (±9.6)	38	0.978
BAS	129	98 (±18)	33	100 (±14)	69	93 (±17)	27	0.171
M-ABC	137	3.5 (2-12)	33	4.5 (2-9.5)	77	7.5 (3-14)	27	0.297
	No. A alleles at *EAAT2* -200/-181							
		1		*2*		*3*		*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	202	0 (0.0%)	16	15 (8.3%)	180	2 (33.3%)	6	0.085
cPVL	199	1 (6.3%)	16	14 (7.9%)	178	0 (0.0%)	5	>0.999
Griffiths	178	100.0 (±9.5)	15	95.6 (±15.3)	157	96.7 (±22.1)	6	0.555
BAS	129	99 (±11)	10	99 (±15)	113	88 (±29)	6	0.249
M-ABC	137	4.8 (0.8-9)	12	5 (2-12)	121	7.5 (1.8-25.8)	4	0.861

Table 2.17. Univariable tests of association between the cytokine SNPs and all outcomes

		***TNFα* -308**					
		GG		*GA*		*AA*	*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	178	6 (6.0%)	100	8 (11.8%)	68	3 (30.0%)	10	**0.043**
cPVL	175	6 (6.1%)	99	6 (9.1%)	66	3 (30.0%)	10	**0.051**
Griffith	156	96.5 (±16.2)	90	95.0 (±12.9)	57	92.4 (±25.5)	9	0.696
BAS	114	99 (±15)	62	95 (±16)	46	105 (±14)	6	0.191
M-ABC	121	4.5 (2.5-11.5)	67	5 (1-11.5)	47	5 (4-27.5)	7	0.637
		***IL1β* -511**					
		CC		*CT*		*TT*	*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	170	10 (11.9%)	84	5 (7.6%)	66	0 (0.0%)	20	0.272
cPVL	167	5 (6.0%)	83	4 (6.3%)	64	1 (5.0%)	20	>0.999
Griffith	152	93.9 (±16.0)	73	98.8 (±13.9)	63	97.1 (±13.9)	16	0.160
BAS	109	94 (±16)	53	103 (±14)	46	100 (±15)	10	**0.017**
M-ABC	118	6.75 (3.3-12)	60	4 (1-9.5)	47	4.5 (3-12.5)	11	0.141
		***IL6* -174**					
		GG		*GC*		*CC*	*p*
	Total n		*n*		*n*		*n*	
Cerebral Palsy	140	3 (6.3%)	48	6 (9.0%)	67	4 (16.0%)	25	0.426
cPVL	137	3 (6.4%)	47	2 (3.1%)	67	4 (16.0%)	25	0.082
Griffith	124	94.0 (±15.1)	44	96.6 (±16.9)	59	98.5 (±13.6)	21	0.519
BAS	89	96 (±18)	32	99 (±16)	40	104 (±14)	17	0.261
M-ABC	96	3.5 (2-11.5)	35	4 (0.5-11.5)	43	5.8 (2.5-9.5)	18	0.831

2.4.4.1 Cerebral Palsy

In the univariable tests (Table 2.18), there was some evidence that *TNFα* -308 was associated with CP (p=0.043). The odds ratio for the heterozygote GA genotype relative to the reference

GG genotype was 2.09 (95% CI 0.60-7.66), whereas the odds ratio for the homozygote AA genotype was 6.71 (95% CI 0.87-39.84). 3 out of 10 children with the *TNFα* -308 AA genotype had CP, which was higher than expected by chance in the *post hoc* analyses (adjusted Pearson residual = 2.27). Of these 3 children, one had diplegia, one had quadriplegia and one non-Caucasian child had hemiplegia. Analysis was repeated in the entire cohort with *TNFα* -308 data, regardless of availability of *EAAT2* genetic data. However, all 29 additional children included in this larger cohort (n=207 vs 178) had the *TNFα* -308 GG reference genotype.

There was no evidence that CP was associated with *IL1β* -511, *IL6* -174, or either of the glutamate transporter variants individually. There was very weak evidence of an association with the number of A alleles at *EAAT2* -200/-181 genotype combination (p=0.085), however numbers were very low in the rarest allele counts, with 2 out of 6 children with 3 A alleles having CP and none of the 16 children with 1 A allele.

Table 2.18. Univariable tests of association between all SNPs and cerebral palsy

Genetic Variant	***Cerebral Palsy - Univariable Tests of Association***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	202	*CC*	50	*CA*	116	*AA*	36	>0.999
		4 (8.0%)		10 (8.6%)		3 (8.3%)		
EAAT2 -181	202	AA	45	AC	116	CC	41	0.690
		5 (11.1%)		10 (8.6%)		2 (4.9%)		
No. A alleles at EAAT2 -200/-181	202	1		2		3	6	0.085
		0 (0.0%)		15 (8.3%)		2 (33.3%)		
TNFα -308	178	GG	100	GA	68	AA	10	**0.043**
		6 (6.0%)		8 (11.8%)		3 (30.0%)		
IL1β -511	170	CC	84	CT	66	TT	20	0.272
		10 (11.9%)		5 (7.6%)		0 (0.0%)		
IL6 -174	140	GG	48	GC	67	CC	25	0.426
		3 (6.3%)		6 (9.0%)		4 (16.0%)		

In the multivariable interaction analyses, the cohort was subdivided into smaller subsets based on genotypes at both *EAAT2* and cytokine SNPs (Table 2.19**,** Figure 2.2**,** Figure 2.3**,** Figure 2.4). Most children (89%) had 2 A alleles at *EAAT2* -200/-181 genotype combination. Only a small number of children had 1 and 3 alleles at *EAAT2*, less than 20 when considering only the children with available CP and cytokine data. None of the children with 1 or 3 A alleles were also homozygote for the risk allele at the cytokine, except for one child with *IL6* -174 CC, and heterozygotes were also rare (n=6-12 depending on the SNP). This precluded assessment of the genotype combinations hypothesised *a priori* as carrying the highest risk.

According to the widely used "Events Per Variable" (EPV) rule of thumb, at least 10 cases are needed for each predictor variable included in a model to avoid generating models that have poor predictive power and are too uncertain (1070-1072). The problems with fitting regression models with low number of events (i.e. CP cases) include inaccuracy of the logit coefficients due to small sample bias, with systematic overestimation of the associations, as well as the issue of separation, whereby a single predictor perfectly separates events from non-events. The current study features two genetic exposures (the number of A alleles at *EAAT2* -200/-181 and a cytokine genotype) and their interaction and would therefore require at least 30 CP cases. This is far more than the 17 CP cases in the complete cases cohort. Moreover, separation is not perfect but substantial, with the vast majority of CP cases having 2 A alleles at *EAAT2* due to rarity of the other combinations with current sample size. On separated data, the maximum likelihood estimation procedure, which estimates the best fitting model of the observed data, can go through the iterations, and fail to converge on an estimate (1073). This was indeed observed when attempting to fit a multivariable regression model. As such, due to the rarity of both CP outcome and risk genetic variants, logistic regression was considered inappropriate with current sample size.

Table 2.19. Combined *EAAT2*-cytokine genotype distributions in relation to cerebral palsy

Cytokine Variant	Genotype	Cerebral Palsy	No. A alleles at *EAAT2 -200/-181*		
			1	2	3
TNFα -308	*GG*	*No*	8	83	3
		Yes	-	6	-
	GA	*No*	6	54	-
		Yes	-	6	2
	AA	*No*	-	7	-
		Yes	-	3	-
IL1β -511	*CC*	*No*	5	68	1
		Yes	-	9	1
	CT	*No*	10	50	1
		Yes	-	4	1
	TT	*No*	-	20	-
		Yes	-	-	-
IL6 -174	*GG*	*No*	6	37	2
		Yes	-	2	1
	GC	*No*	5	56	-
		Yes	-	5	1
	CC	*No*	-	20	1
		Yes	-	4	-

Figure 2.2. Combined *EAAT2-TNFα* genotype distributions in relation to cerebral palsy

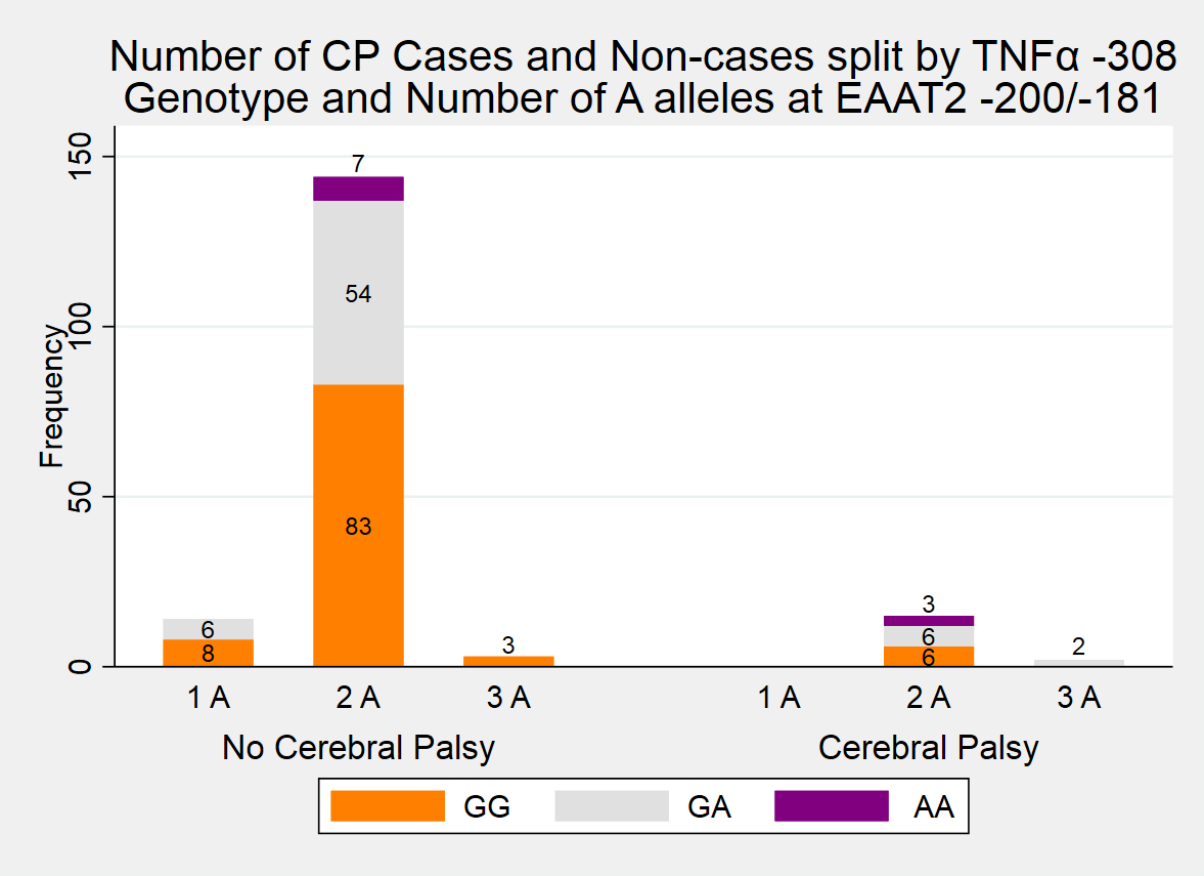


Figure 2.3. Combined *EAAT2-IL1β* genotype distributions in relation to cerebral palsy

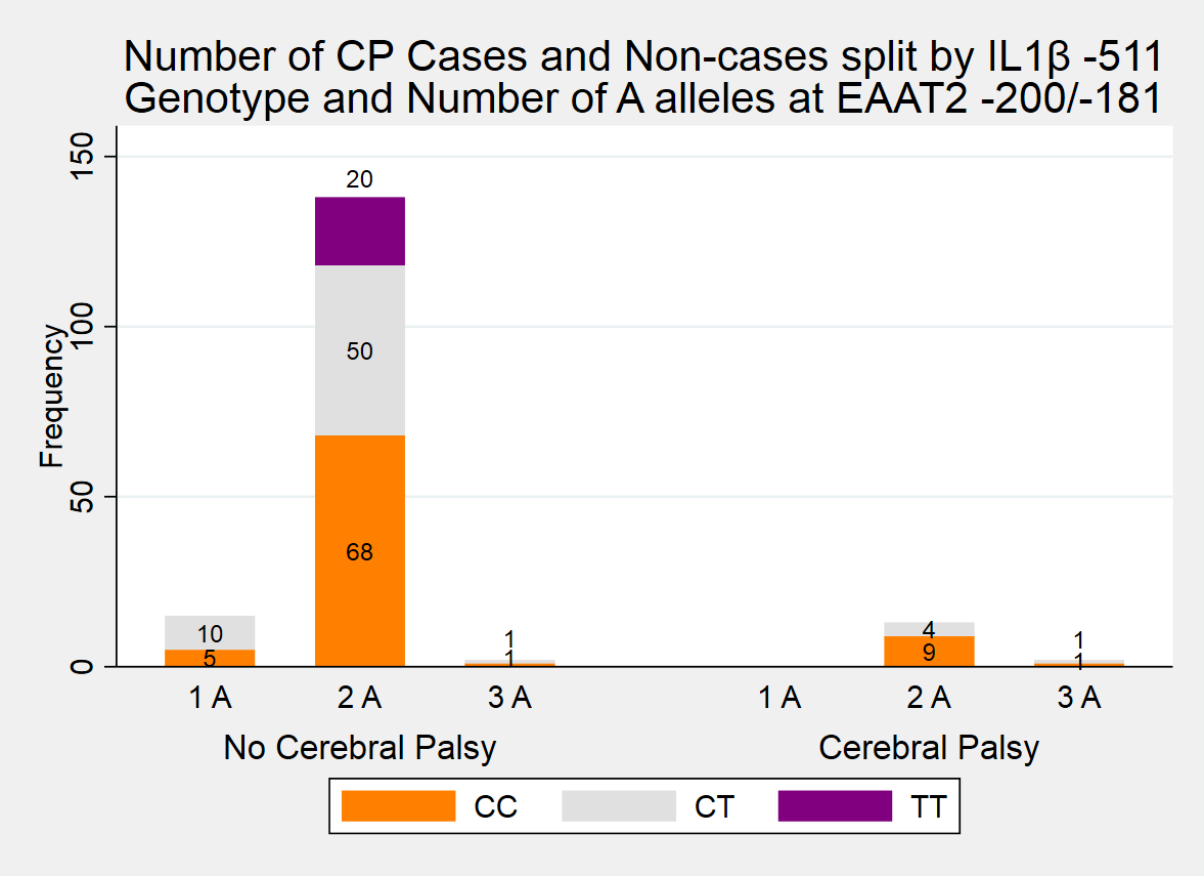
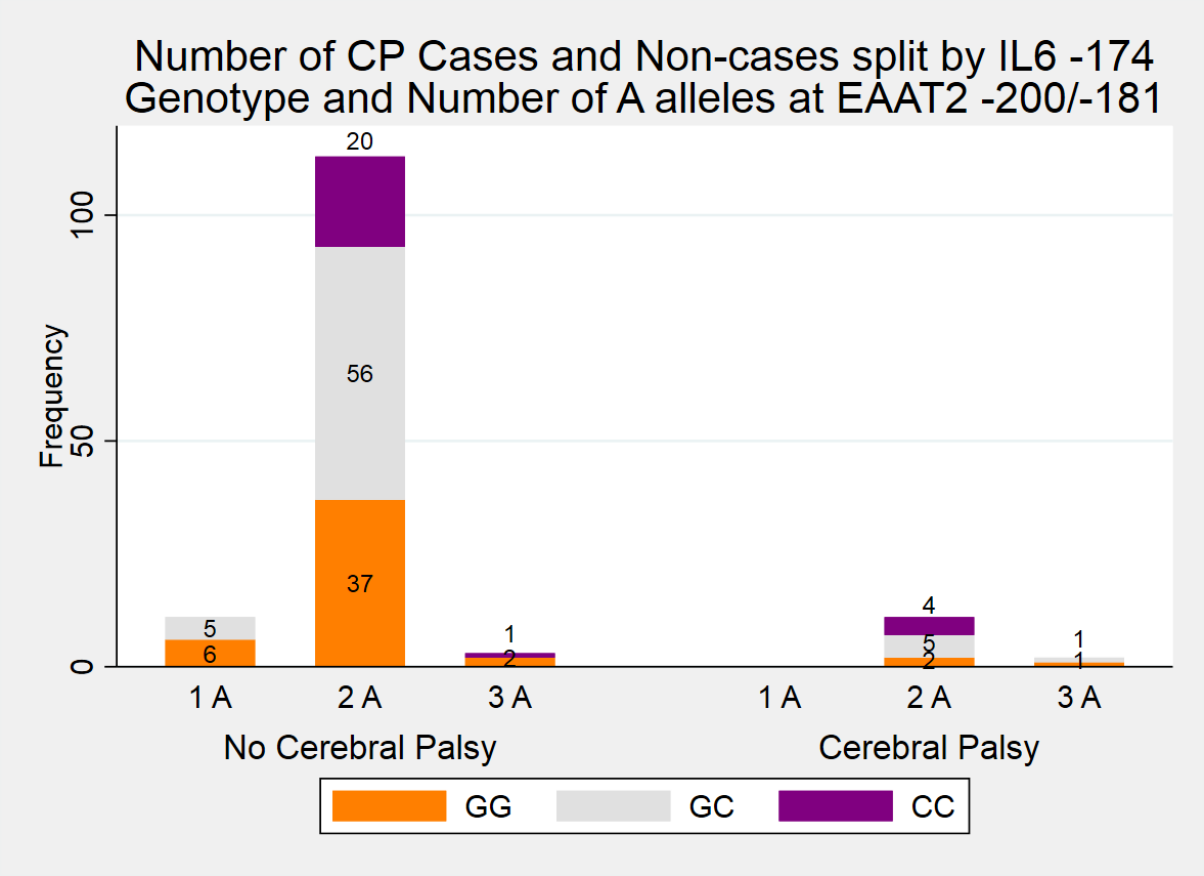


Figure 2.4. Combined *EAAT2-IL6* genotype distributions in relation to cerebral palsy



An example of how logistic regression could be carried out with a larger sample size is given for *EAAT2* and *TNFα* in the Appendix (Table 8.2). Informally, it can be observed that none of the children with 1 A allele at *EAAT2* have CP, whereas both children with 3 A alleles and CP also have the *TNFα* heterozygote genotype. Whilst in agreement with the *a priori* hypothesis, larger studies are needed with greater number of CP cases and rare genotype combinations before regression modelling can be applied with precision. As such, statistically sound inferences about the genetic interaction effects of the glutamate transporter and cytokine variants on CP risk cannot be drawn at this stage.

To assess potential effect modification, children with CP were stratified by both genotype and perinatal inflammatory exposures (Table 2.20). The number of children with CP does not always amount to 17 because of missing genetic and/or clinical data. Stratified numbers were too low for statistical testing of heterogeneity of effects across strata. Informally, it can be observed that two of the children with CP had multiple inflammatory exposures and multiple cytokine risk genotypes: one child with diplegia (indicated as *) had a history of both maternal infection and neonatal sepsis and had *TNFα* -308 AA, *IL1β* -511 CC and *IL6* -174

GC. Another child with quadriplegia (indicated as ^) had a history of both necrotising enterocolitis and neonatal sepsis and had *TNFα* -308 AA and *IL6* -174 GC genotype (missing data for *IL1β* -511). Both children had 2 A alleles at *EAAT2* -200/-181. Stratification by SGA status was not appropriate in this cohort, as no children identified as small for gestational age in the complete cases cohort had CP.

Table 2.20. Stratification of the children with cerebral palsy by both genotype and perinatal inflammatory exposures

Genotype	**Number of Children with CP and Perinatal Inflammatory Exposures**					
	Maternal Infection		**Neonatal Infection**		**Necrotising Enterocolitis**	
	No	*Yes*	*No*	*Yes*	*No*	*Yes*
TNFα -308						
GG	5	1	3	3	6	-
GA	7	-	5	3	8	-
AA	2	1*	-	3*^	2	1^
IL1β -511						
CC	8	2*	6	4*	10	-
CT	4	-	1	4	5	-
TT	-	-	-	-	-	-
IL6 -174						
GG	3	-	1	2	3	-
GC	4	1*	3	3*^	5	1^
CC	3	1	2	2	4	-
No. A alleles EAAT2 -200/-181						
1	-	-	-	-	-	-
2	13	2*	6	9*^	14	1^
3	1	-	2	-	2	-

** and ^ - two children with multiple inflammatory exposures*

2.4.4.2 Cystic Periventricular Leukomalacia

In the complete cases cohort, 17 children had CP and 15 newborns had cPVL at birth. Approximately half (n=8) of the children with CP had cPVL at birth. Of these, 3 (19%) also had severe intraventricular haemorrhage (IVH grade 3 or 4). Indeed, all CP cases with severe IVH also had cPVL in this cohort. Of the remaining 9 children with CP, one had moderate injury (persistent periventricular echodensities), 7 had normal ultrasound data and one had no ultrasound data.

In the univariable tests (Table 2.21), there was some evidence that *TNFα* -308 was associated with cPVL (p=0.051). The odds ratio for the heterozygote GA genotype relative to the reference GG genotype was 1.55 (95% CI 0.39-6.08), whereas the odds ratio for the homozygote AA genotype was 6.64 (95% CI 0.86-39.42). 3 out of 10 children with the *TNFα* -308 AA genotype had cPVL, which was higher than expected by chance in the *post hoc* analyses (adjusted Pearson residual = 2.49). These were the same 3 children with *TNFα* -308 AA who had CP. All three children also had some form haemorrhage: one child developed quadriplegia and had severe intraventricular haemorrhage at birth (IVH grade 3 or 4); one child developed diplegia and had moderate IVH (grade 2) and one child with hemiplegia had a mild subependymal haemorrhage (grade 1).

There was no evidence that cPVL was associated with *IL1β* -511 or the glutamate transporter variants in the univariate analyses. On the other hand, there was very weak non-significant evidence of an association with *IL6* -174 (p=0.082). The complete cases cohort for *IL6* -174 and cPVL outcome was the smallest (n=137), with only 2-4 cPVL cases for each *IL6* -174 genotype. When the analysis was repeated in the whole cohort with *IL6* -174 data, regardless of availability of *EAAT2* genetic data (n=157), the number of children with cPVL for each genotype was still very low (n=3-4) and there was no longer any evidence of association.

Table 2.21. Univariable tests of association between all SNPs and cystic periventricular leukomalacia

Genetic Variant	***Cystic Periventricular Leukomalacia - Univariable Tests of Association***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	199	CC	50	CA	114	AA	35	0.812
		3 (6.0%)		10 (8.8%)		2 (5.7%)		
EAAT2 -181	199	AA	45	AC	113	CC	41	>0.999
		3 (6.7%)		9 (8.0%)		3 (7.3%)		
No. A alleles at EAAT2 -200/-181	199	1	16	2	178	3	5	>0.999
		1 (6.3%)		14 (7.9%)		0 (0.0%)		
TNFα -308	175	GG	99	GA	66	AA	10	**0.051**
		6 (6.1%)		6 (9.1%)		3 (30.0%)		
IL1β -511	167	CC	83	CT	64	TT	20	>0.999
		5 (6.0%)		4 (6.3%)		1 (5.0%)		
IL6 -174	137	GG	47	GC	67	CC	25	0.084
		3 (6.4%)		2 (3.1%)		4 (16.0%)		

A sensitivity analysis was performed with a new variable describing any moderate to severe white matter injury detectable through ultrasound (Table 2.22). Alongside cPVL (n=23), this comprised persistent periventricular echodensities not evolving into cysts (n=10), and ventricular dilatation (n=11), which can be caused by atrophy of the periventricular white matter in diffuse white matter injury or with microscopic glial scars not visible with ultrasound. Inclusion of these more moderate forms of white matter injury did not affect the size of the complete cases cohort (n=157) but increased the number of cases (n=44). The association with *TNFα* -308 was no longer supported (p=0.631), whereas the evidence supporting *IL6* -174 strengthened (p=0.052). *Post hoc* residual analysis suggested that the reference CC genotype was associated with a significantly higher proportion of moderate to severe white matter injury (37.5%, adjusted Pearson residual = 2.37).

Table 2.22. Sensitivity analysis of the SNP effects on risk of any moderate to severe white matter injury

Outcome	Exposure							
		Total n		*n*		*n*		*p*
Moderate to severe white matter injury	*TNFα -308*		*GG*		*GA*		*AA*	
		202	19 (16.0%)	119	12 (16.7%)	72	3 (27.3%)	11	0.631
	IL1β -511		CC		CT		TT	
		194	12 (12.8%)	94	10 (13.0%)	77	3 (13.0%)	23	0.999
	IL6 -174		GG		GC		CC	
		157	5 (9.8%)	51	10 (13.3%)	75	9 (29.0%)	31	**0.052**
	EAAT2 -200		CC		CA		AA	
		201	6 (11.8%)	51	19 (16.5%)	115	6 (17.1%)	35	0.702
	EAAT2 -181		AA		AC		CC	
		201	5 (11.1%)	45	19 (16.5%)	115	7 (17.1%)	41	0.659
	No. A alleles at EAAT2 -200/-181		1		2		3	
		201	2 (11.8%)	17	29 (16.2%)	179	0 (0.0%)	5	>0.999

As for CP, cPVL is a rare outcome, and the study is especially underpowered for multivariate interaction analysis (Table 2.23).

Table 2.23. Combined *EAAT2*-cytokine genotype distributions in relation to cystic periventricular leukomalacia

Cytokine Variant	**Genotype**	**cPVL**	**No. A alleles at *EAAT2 -200/-181***		
			1	2	3
TNFα -308	*GG*	*No*	7	83	3
		Yes	1	5	-
	GA	*No*	6	53	1
		Yes	-	6	-
	AA	*No*	-	7	-
		Yes	-	3	-
IL1β -511	*CC*	*No*	5	71	2
		Yes	-	5	1
	CT	*No*	10	49	1
		Yes	-	4	-
	TT	*No*	-	19	-
		Yes	-	1	-
IL6 -174	*GG*	*No*	6	35	3
		Yes	-	3	-
	GC	*No*	5	58	
		Yes	-	2	-
	CC	*No*	-	20	1
		Yes	-	4	-

2.4.4.3 Griffiths Neurodevelopmental Scales at 2y

In the univariable tests (Table 2.24), there was no evidence that any of the genetic variants was associated with the Griffiths aggregate general quotient, assessing movement, cognition, perception, and communication at 2 years of age.

Table 2.24. Univariable tests of association between all SNPs and the Griffiths aggregate general quotient at 2 years

Genetic Variant	***Griffiths Developmental Score (2y) - Univariable Tests of Association***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	178	CC	46	CA	99	AA	33	0.936
		96.7 (±16.6)		95.7 (±16.0)		95.7 (±9.7)		
EAAT2 -181	178	AA	42	AC	98	CC	38	0.978
		95.6 (±18.3)		96.2 (±15.5)		95.9 (±9.6)		
No. A alleles at EAAT2 -200/-181	178	1	15	2	157	3	6	0.555
		100.0 (±9.5)		95.6 (± 15.3)		96.7 (±22.1)		
TNFα -308	156	GG	90	GA	57	AA	9	0.696
		96.5 (±16.2)		95.0 (±12.9)		92.4 (±25.5)		
IL1β -511	152	CC	73	CT	63	TT	16	0.160
		93.9 (±16.0)		98.8 (±13.9)		97.1 (±13.9)		
IL6 -174	124	GG	44	GC	59	CC	21	0.519
		94.0 (±15.1)		96.6 (±16.9)		98.5 (±13.6)		

Linear regression modelling the effect of the increasing number of A alleles at *EAAT2* on the Griffiths score would assume a linear relationship between the two variables. However, the low number of children with 1 and 3 A alleles and the lack of children with 0 and 4 A alleles did not allow to reasonably make this assumption (Figure 2.5). Additionally, there were few children with available Griffiths data and non-reference *EAAT2*-cytokine genotype combinations (n<10) (Table 2.25). Therefore, progression to linear regression modelling was deemed inappropriate.

Figure 2.5. Scatterplot of the Griffiths score at 2 years by the number of A alleles at *EAAT2* -200/-181

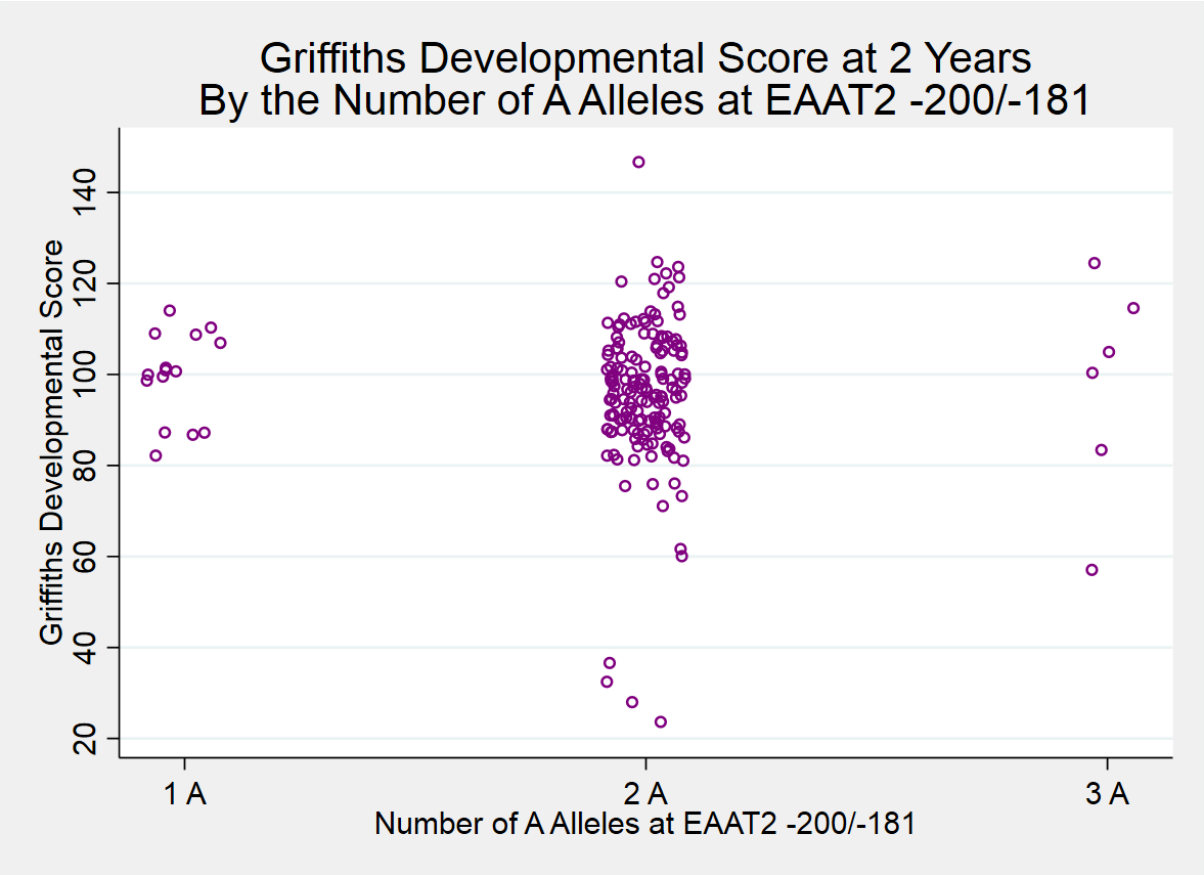


Table 2.25. Combined *EAAT2*-cytokine genotype distributions in relation to the number of children with the Griffiths score at 2 years

Cytokine	Genotype	*EAAT2(#A) -200/-181*		
		1	*2*	*3*
	GG	8	79	3
***TNFα* -308**	*GA*	5	50	2
	AA	-	9	-
	CC	5	66	2
***IL1β* -511**	*CT*	9	52	2
	TT	-	16	-
	GG	5	36	3
***IL6* -174**	*GC*	5	53	1
	CC	-	20	1

2.4.4.4 British Ability Scales Cognitive Score at 5y

Children with missing BAS cognitive data at 5 years were more likely to have had a CP diagnosis at 2 years (p=0.042), but not necessarily a lower Griffiths score (p=0.179).

In the univariable tests (Table 2.26), there was some evidence that the BAS score was associated with *IL1β* -511 (p=0.017). *Post hoc* analysis revealed that the BAS score was significantly lower in children with the reference CC genotype compared to the CT genotype (94 (±16) vs 103 (±14), p=0.003). Evidence was weaker for CC vs the rarest TT genotype (100 (±15), p=0.193). The difference in means between CT and CC was 8.93 (2.84-15.02) and the difference in means between TT and CC was 5.52 (-4.90-15.94). Repeating the analysis in the whole *IL1β* -511 complete cases cohort, regardless of availability of EAAT2 genetic data, increased sample size (n=127 vs 109) and included 2 additional children with TT genotype. In this analysis, the evidence for a difference between CC and TT genotypes strengthened (p=0.078). There was also very weak evidence of an association between the BAS score and *EAAT2* -200 (p=0.084).

Table 2.26. Univariable tests of association between all SNPs and the BAS score at 5y

Genetic Variant	***British Ability Scales (BAS) Cognitive Score (5 y) - Univariable Tests of Association***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	129	CC	34	CA	71	AA	24	0.084
		99 (± 15)		100 (± 15)		92 (± 18)		
EAAT2 -181	129	AA	33	AC	69	CC	27	0.171
		98 (±18)		100 (± 14)		93 (± 17)		
No. A alleles at EAAT2 -200/-181	129	1	10	2	113	3	6	0.249
		99 (± 11)		99 (± 15)		88 (± 29)		
TNFα -308	114	GG	62	GA	46	AA	6	0.191
		99 (± 15)		95 (± 16)		105 (± 14)		
IL1β -511	109	CC	53	CT	46	TT	10	**0.017**
		94 (± 16)		103 (± 14)		100 (± 15)		
IL6 -174	89	GG	32	GC	40	CC	17	0.261
		96 (± 18)		99 (± 16)		104 (± 14)		

A sensitivity analysis explored association between *IL1β* -511 and the *EAAT2* variants and the BAS subscales, i.e. verbal ability, non-verbal reasoning ability and spatial ability (Table 2.27). There was increasing evidence that *IL1β* -511 was associated with the non-verbal reasoning subscale (p=0.003). Specifically, children with the reference CC genotype had lower non-verbal reasoning scores than both the CT heterozygotes (p=0.013) and the TT homozygotes for the minor allele (p=0.008). There was also some evidence that *EAAT2* -200 and -181 were both associated with the verbal subscale (-200: p=0.030; -181: p=0.024). Specifically, having two copies of the minor allele at either variant was associated with a lower verbal subscale score than having one copy (-200: p=004; -181: p=0.002) or none (-200: p=0.009; -181: p=0.013). There was no association with the number of A alleles at *EAAT2*, though all three subscale scores were nominally lower in the 6 children with 3 A alleles.

Table 2.27. Sensitivity analysis of the effects of *IL1β* -511, *EAAT2* -200 and -181 on the subscales of the BAS score at 5 years

BAS Subscales		Genetic Variant							p
		n		*n*		*n*		*n*	
Verbal Ability	*IL1β -511*	134	CC 100 (±13)	65	CT 103 (±14)	57	TT 99 (±12)	12	0.339
	EAAT2 -200		CC 102 (±12)	35	CA 102 (±14)	78	AA 94 (±15)	25	**0.028**
	EAAT2 -181	138	AA 102 (±14)	33	AC 102 (±13)	76	CC 94 (±14)	29	**0.022**
	No. A alleles at EAAT2 -200/-181		1 100 (±10)	12	2 101 (±14)	120	3 96 (±19)	6	0.726
Non-Verbal Reasoning Ability	*IL1β -511*	129	CC 94 (±15)	62	CT 101 (±16)	55	TT 110 (±20)	12	**0.003**
	EAAT2 -200		CC 100 (±15)	34	CA 99 (±17)	73	AA 92 (±15)	24	0.132
	EAAT2 -181	131	AA 100 (±18)	33	AC 100 (±15)	70	CC 92 (±16)	28	0.079
	No. A alleles at EAAT2 -200/-181		1 93 (±17)	11	2 99 (±15)	114	3 91 (±26)	6	0.199
Spatial Ability	*IL1β -511*	126	CC 95 (±18)	61	CT 99 (±17)	53	TT 99 (±11)	12	0.399
	EAAT2 -200		CC 99 (±14)	33	CA 97 (±16)	73	AA 94 (±20)	23	0.641
	EAAT2 -181	129	AA 97 (±17)	32	AC 97 (±16)	71	CC 97 (±17)	26	0.980
	No. A alleles at EAAT2 -200/-181		1 102 (±10)	10	2 97 (±16)	113	3 84 (±29)	6	0.109

As for the Griffiths score, progression to linear regression was deemed inappropriate due to the inability to assume linearity between the increasing number of A alleles at *EAAT2* and the BAS score (Figure 2.6) and the small number of children with available BAS data and non-reference *EAAT2*-cytokine genotype combinations (n<10) (Table 2.28).

An example of how linear regression could be carried out with a larger sample size and reasonable linear assumptions is given for *EAAT2* and *TNFα* in the Appendix (Table 8.3). Plots of model predictions could be used to illustrate the predicted effect of the increasing number of A alleles at *EAAT2* on the BAS score by cytokine risk genotypes (Figure 8.1).

Figure 2.6. Scatterplot of the BAS score at 5 years by the number of A alleles at *EAAT2* -200/-181

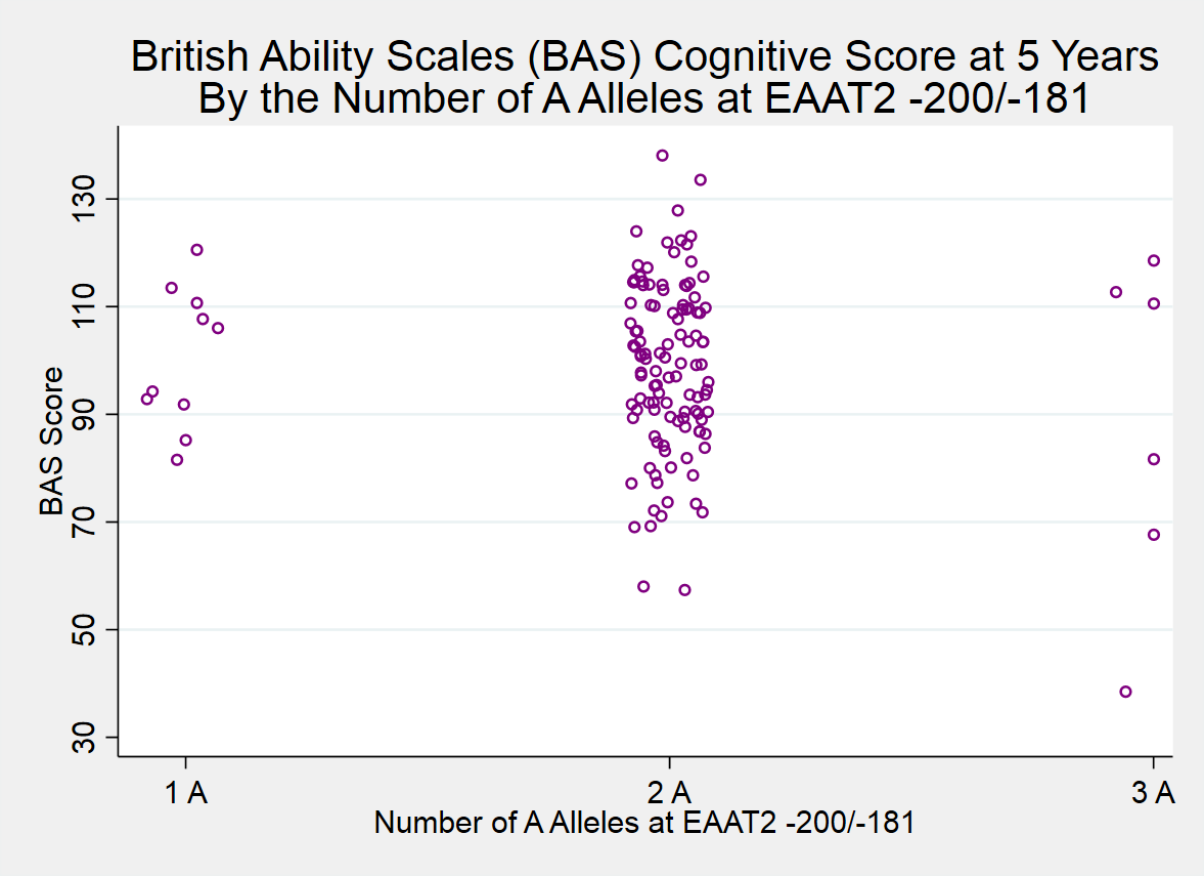


Table 2.28. Combined *EAAT2*-cytokine genotype distributions in relation to the number of children with the BAS score at 5 years

Cytokine	Genotype	*EAAT2(#A) -200/-181*		
		1	*2*	*3*
	GG	5	54	3
***TNFα* -308**	*GA*	4	40	2
	AA	-	6	-
	CC	3	48	2
***IL1β* -511**	*CT*	7	37	2
	TT	-	10	-
	GG	4	25	3
***IL6* -174**	*GC*	4	35	1
	CC	-	16	1

2.4.4.5 Movement Assessment Battery for Children Motor Score at 5y

Children with missing data for the M-ABC score at 5 years were more likely to have had a CP diagnosis at 2 years (p=0.003). This is in part explained by the fact that children with moderate/severe CP are not required to undertake the M-ABC assessment. Additionally, some of the children with moderate/severe impairments may have been lost-to-follow-up. In the univariable tests (Table 2.29), there was no evidence that any of the genetic variants was associated with the M-ABC score, assessing fine and gross motor skills, balance, and coordination at 5 years of age.

Table 2.29. Univariable tests of association between all SNPs and the M-ABC score at 5 years

Genetic Variant	***Movement Assessment Battery for Children (M-ABC) Impairment Score (5 y) - Univariable Tests of Association***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	137	CC	35	CA	81	AA	21	0.440
		3.5 (0.5-10)		5 (2-11.5)		7 (3-12.5)		
EAAT2 -181	137	AA	33	AC	77	CC	27	0.297
		3.5 (2-12)		4.5 (2-9.5)		7.5 (3-14)		
No. A alleles at EAAT2 -200/-181	137	1	12	2	121	3	4	0.861
		4.8 (0.8-9)		5 (2-12)		7.5 (1.8-25.8)		
TNFα -308	121	GG	67	GA	47	AA	7	0.637
		4.5 (2.5-11.5)		5 (1-11.5)		5 (4-27.5)		
IL1β -511	118	CC	60	CT	47	TT	11	0.141
		6.75 (3.3-12)		4 (1-9.5)		4.5 (3-12.5)		
IL6 -174	96	GG	35	GC	43	CC	18	0.831
		3.5 (2-11.5)		4 (0.5-11.5)		5.8 (2.5-9.5)		

As for all other outcomes, regression modelling was deemed inappropriate due to the small number of children with M-ABC data and non-reference *EAAT2*-cytokine genotype combinations (n<10) (Table 2.30). With larger sample sizes, a generalised linear model with

gamma distribution (reflecting the positive skew of the impairment score) could be used to model univariable and multivariable associations (see Appendix, Table 8.4).

Table 2.30. Combined *EAAT2*-cytokine genotype distributions in relation to the M-ABC score at 5 years

Cytokine	Genotype	No. A alleles at *EAAT2* -200/-181		
		1	*2*	*3*
	GG	7	58	2
***TNFα* -308**	*GA*	4	42	1
	AA	-	7	-
	CC	4	54	2
***IL1β* -511**	*CT*	8	38	1
	TT	-	11	-
	GG	5	28	2
***IL6* -174**	*GC*	5	38	-
	CC	-	17	1

In the attempt to increase power, a sensitivity analysis was carried out including the 18 children with CP at 2 years old who did not have a M-ABC score, by assigning them an arbitrary value of 17, i.e. the M-ABC threshold for definite movement impairment (1066). Univariable tests were repeated within each variant's complete cases cohort (Table 2.31). Once again, there was no evidence of association between any of the variants and the M-ABC score.

Table 2.31. Sensitivity analysis of the SNP effects on the M-ABC score at 5 years

Genetic Variant	***Movement Assessment Battery for Children (M-ABC) Impairment Score (5 y) - Univariable Tests of Association (sensitivity analysis including children with missing M-ABC and CP)***							
	Total n		*n*		*n*		*n*	*p*
EAAT2 -200	149	CC 5 (1-17)	39	CA 5 (2-13)	88	AA 7.3 (3-14)	22	0.607
EAAT2 -181	149	AA 6.5 (2-17)	37	AC 5 (2-12)	85	CC 7.5 (3-14)	27	0.450
No. A alleles at EAAT2 -200/-181	149	1 4.8 (0.8-9)	12	2 5.5 (2-13.8)	132	3 11.5 (3.5-17)	5	0.631
TNFα -308	152	GG 5 (3-12)	86	GA 7 (1-12)	57	AA 6.5 (4-17)	9	0.425
IL1β -511	147	CC 6.5 (3-16)	75	CT 4 (1-12)	59	TT 4.5 (3-9)	13	0.276
IL6 -174	116	GG 3.8 (2-12.5)	40	GC 4.5 (1-13)	55	CC 5 (2.5-9.5)	21	0.926

2.5 Discussion

This exploratory study set out to assess whether candidate genetic variants regulating the expression of key pro-inflammatory cytokines and glutamate transporter interact with each other and affect the risk of brain injury and neurodevelopmental impairment in newborns born at or before 32 weeks of gestation. Due to low frequencies of some of the genetic variants and relatively rare outcomes, SNP-SNP interaction analyses were not appropriate,

and the analysis was redesigned to univariable associations only, assessing the effect of each variant independently. The main finding was that genetic variants regulating the expression of key cytokines *TNFα* and *IL1β* may increase the risk of CP and cognitive impairment at pre-school age in children born very preterm.

2.5.1 Genetic models

In exploratory genetic association studies, the underlying mode of inheritance is often not known and pragmatic decisions have to be made about reasonable assumptions in the choice of genetic models. A dominant model (Aa+aa vs AA) assumes that one copy of the minor allele is sufficient for an effect. A recessive model (aa vs Aa+AA) assumes that both copies are needed. A co-dominant additive genetic model (AA vs Aa vs aa) assumes that having two copies of the minor allele has twice the effect of having one copy; this model underlies the linear trend analysis used for the increasing number of A alleles at *EAAT2* -200/-181. The genotypic analyses chosen here consider each genotype as a separate level, without requiring any assumptions about the mode of inheritance, which can instead be argued *a posteriori* for future work based on effect sizes (1074).

One of the largest candidate SNP studies to date, assessing 39 candidate variants in 587 cases and 1154 controls from Australia, used allelic analyses (a vs A, assuming HWE), followed by dominant, recessive, and genotypic analyses whenever deviation from the additive model was observed (162). This leads to a further increase in multiple comparisons, in a study design already featuring multiple genetic exposures. Most candidate SNP studies of CP to date have used genotypic analyses, making the current study more directly comparable to the wider literature. By using a genotypic approach, we have also kept with recommendations made by the Human Genome Epidemiology Network (HuGENet), a global collaborative effort established by the U.S. Centers for Disease Control (CDC): “where possible, separate effect estimates for heterozygotes and for homozygotes be presented to allow readers to judge for themselves issues around the likely mode of inheritance and the choice of model” (1075). The downside of this approach is reduced power, which is best addressed by replicating any findings in a larger sample.

2.5.2 Missing data

Missing genetic data can be due to missing blood spots or failure of the DNA extraction or amplification processes. The molecular methodologies used by the original authors have known strengths and weaknesses. Manual methods for DNA extraction, such as the boiling

method followed by phenol extraction used for the cytokine variants, are usually robust for simple PCR screening (1030). However, yield and purity can be more variable than with commercial kits used for the glutamate transporter variants (911). This may have contributed to the much smaller complete cases subsets for the cytokine variants compared to the glutamate transporter variants, especially for *IL6* -174 and *IL1β* -511. Moreover, while both RFLP and pyrosequencing are relatively robust genotyping methods, comparative studies have reported higher accuracy for pyrosequencing (1076-1078). For example, 4 out of 46 *TNFα*-308 GG homozygotes were misclassified as heterozygotes with RFLP compared to pyrosequencing in a cohort of traumatic and burn injury patients (1079). Accuracy of the pyrosequencing method used for the glutamate transporter variants was further supported by evidence of complete concordance with the 10% data re-genotyped by Sanger sequencing, which is the gold standard for sequencing accuracy in low throughput analyses (911). As such, cytokine data may be more likely not only to be missing but also to be less accurate compared to the glutamate transporter. This type of limitation is unavoidable when using historical data. For a future prospective study, pyrosequencing remains suitable when investigating multiple variants in the same region (e.g. the glutamate transporter variants) in small to medium-sized projects. As it is relatively time-, labour- and cost-intensive, other methods may be more suitable if looking at many independent variants in different regions of the genome (e.g. TaqMan fluorescent probes) (1080).

Outcome data were also incomplete, and data was not missing completely at random (MCAR), as children with missing motor and cognitive outcomes at 5 years were more likely to have had CP at 2 years. It is also possible that 5 year outcomes were missed for children diagnosed with CP at a later age (28). This suggests that the children with the most severe motor disabilities were more likely to have been lost to follow up, leading to sub-selection of the children with milder motor impairment. This means not only a loss of power but also likely underestimation of the genetic effects for the 5 year outcomes. The non-attendance for follow-up may be due to several reasons, including the socioeconomic status of the mother/family, poor parental health, maternal age, maternal education and distance to hospital; perinatal and developmental characteristics of the child may also affect retainment in the study, since it has been shown that parents of the sickest newborns may be more likely to attend follow-up visits and take part in relevant research in the long-term (223, 1081-1084).

Moreover, children with moderate/severe CP are not required to undertake the M-ABC assessment. Given the association between dependent outcome measures, children with missing M-ABC who were diagnosed with CP at 2 years were assigned an arbitrary value corresponding to the M-ABC threshold for "definite motor problems" (1066). This is a consistent approach with large population studies which have defined motor impairment as a diagnosis of CP and/or M-ABC score 17-40 (<5th centile) (215). A more refined approach would be to collect data from the Gross Motor Function Classification System (GMFCS) (1085, 1086). This would allow stratification by CP severity, with the maximum score assigned to children with the most severe forms of CP, who were not able to start or complete the M-ABC assessment.

2.5.3 Characteristics of the study population

The rates of CP and cPVL (around 8%) are comparable to other studies carried out in the 90s (115, 1087-1089). Just under half of the children with CP had a normal ultrasound scan at birth, which is considerably higher than the 5-7% previously identified by Kapitanovic Vidak and colleagues (908) and De Vries and colleagues (96) and the third identified by Ancel and colleagues (350). In the future, serial ultrasound could be collected to assess development of injury in the neonatal period (595), alongside MRI data for a more comprehensive assessment of brain injuries (1090). The mean values for the neurodevelopmental outcomes are slightly lower than the expected population mean of 100, though within the normal range (+1SD). This is in line with finding from the original APIP study that very preterm newborns tend to have lower neurodevelopmental scores compared to the general population (275, 276). The trial arm allocation was not reported to confound any of the previous analyses (1030).

The *TNFα* -308 minor A allele was significantly more frequent in this cohort compared to the 1000 Genomes European subset. A potential explanation is that fetal *TNFα* -308 (or a variant in LD) increases the risk of preterm birth and is therefore more frequent in this selected very preterm cohort. Maternal *TNFα* -308 has previously been reported in association with preterm birth, including in combination with fetal -308 (1091-1094). However, findings have been inconsistent, with several studies, including a meta-analysis, reporting no association (1095-1101) or association with two haplotypes containing the G allele rather than the minor A allele (1102). While association with -308 is controversial, other variants in *TNFα* and its receptors have been found to differ significantly by ethnicity and this has been proposed to contribute to ethnic disparities in rates of preterm birth (1103). This is an important aspect to consider when interpreting studies of CP since, for example, the higher prevalence of CP in

black newborns seems to be largely mediated by a disparity in the risk of preterm birth which cannot be explained solely by sociodemographic factors and access to healthcare (188, 1104-1107).

Genetic differences across populations are at the basis of stratification by ethnicity in genetic association studies. This design has the benefit of allowing for both scenarios in which ethnicity is an effect modifier (i.e. the genetic effect is different depending on ethnicity) or a confounder (i.e. population stratification: the genetic effect is the same in all ethnicities and ethnicity is independently associated with both allele frequencies and disease outcomes), although confounding can also be handled by adjustment at the analysis stage (1108). Practically, it is difficult to distinguish between these two scenarios unless the risk alleles have been first robustly identified in non-stratified samples, which is not the typical scenario of exploratory studies. Additionally, in cohort studies, ethnicity is not uniformly distributed and *post hoc* stratification often lacks statistical power. Our study suggests that the discordant *EAAT2* genotypes hypothesised to be detrimental are more frequent in non-Caucasians. As expected, the current sample does not allow a more in-depth analysis of ethnicity effects, as only 18 children were non-Caucasian, with no available detailed ethnicity information. Future studies on variants suspected to have different allele frequencies in different populations should be designed, where possible, to either focus on each population separately or to have sufficient power for *post hoc* stratification.

2.5.4 The study is underpowered to assess the combinations of glutamate transporter variants and their interaction with cytokine variants

GWAS of complex traits have highlighted that only a portion of heritability estimated by twin and family studies can be explained by additive genetic variance, i.e. the combined effect of each indiviual variant. The unexplained heritability has been attributed to multiple factors, including rare and structural variants, epigenetic factors, gene-environment and gene-gene interactions (1109, 1110). Exploring gene-gene interactions can get us closer to understanding the complex genomic architecture underpinning neurological and neurodevelopmental traits. However, these studies are known to be problematic in terms of both interpretation and power (1111-1113).

The issue of interpretation is more obvious in GWAS designs, whereby the biological relevance of statistically significant interaction found by scanning throughout the genome without any prior hypothesis may be less clear. A statistical interaction is simply a mathematical deviation from linearity in a model of the effect of two exposures on the log-

odds of disease, and does not carry biological relevance *per se* (1114). However, a substantial body of clinical and experimental evidence supports a biological mechanism of potentiation between glutamate excitotoxicity and inflammation, backing the hypothesis of genetic interactions. On the other hand, interaction studies typically suffer from power issues compared to main effects only analyses, since the sample is subdivided into more subsets, each with lower counts. This precluded assessment of the *EAAT2* SNP-cytokine SNP interaction hypothesis in this study, due to the combination of three factors with a taxing effect on power:

- CP is a relatively rare binary outcome. This is an issue inherent to all studies of CP, which have historically suffered from power issues. Here, we have focused on a very preterm population since failing to assess different pathophysiologies of CP separately may also result in reduced power (1115). Despite the APIP cohort being one of the largest prospective very preterm cohorts with CP data, sample size remains limited, with 17 CP cases amongst 202 very preterm survivors
- Most (89%) children in the sample had 2 A alleles at the *EAAT2* genotype combination, while all other *EAAT2* genotype combinations were rare (16 children with 1 A allele, 6 children with 3 A alleles) or absent (no children with 0 and 4 A alleles). This translated in little variability in the glutamate genetic exposure and limited statistical power already at the univariable stage
- The risk genotype combinations at the glutamate transporter were even more rarely found in combination with the risk genotypes at the cytokines, further exacerbating the power issue in multivariable analyses of interaction effects

Due to the power issue, the study was scaled down to univariable analyses, exploring the independent effect of each variant on neurodevelopmental outcomes. Statistical adjustment for multiple comparison was not considered necessary here, given that this is an exploratory study based on an *a priori* hypothesis for candidate pathways known to interact in the developing brain. False negatives are costly in exploratory studies and the best approach is to interpret with caution and replicate the findings in new datasets (1116). Similarly, other candidate gene studies have chosen to not correct for multiple comparison, while others have shown near total loss of significant findings after correction with even larger sample sizes (162, 901, 917, 1115). Notably, a large meta-analysis of 17 candidate SNPs previously implicated in CP with over 2,500 CP cases and 4,400 controls only confirmed an association with *IL6* -174 (912). The best approaches to address the potential issues of false positives and

negatives and identify robust genetic associations in the future remain to increase sample size by recruitment and collaborations and replicate findings in independent cohorts. This will ideally include different ethnicities, since variable allele frequencies are known to contribute to power issues and poor replication (1117), and especially given the finding that *EAAT2* discordant genotypes may be more common in non-Caucasian ethnicies (911). Future studies focusing specifically on interaction effects should consider the known issue of scale when interpreting interactions, since an effect may appear larger in one group on the difference (additive) scale but smaller on the ratio (multiplicative) scale and therefore both should be reported for completeness (1118, 1119).

2.5.5 TNFα -308 is associated with cPVL at birth and cerebral palsy at 2y

The primary outcome of CP was associated with the *TNFα* -308 AA genotype, with 3 out of 10 (30%) children with AA having CP compared to 6 in 100 (6%) children with GG. Previous smaller case-control studies found no association with CP in very preterm newborns (910) or a small protective effect, including in children with cPVL at birth (908). In larger South Australian case-control studies, the variant was reported to have both a protective effect in quadriplegia at all gestational ages and in moderately preterm newborns when analysed with a recessive model (AA vs GG + GA) (162, 917); on the other hand, the variant had a detrimental effect in hemiplegia in very preterm newborns for children with GA or AA genotypes vs reference GG genotype (906). This study did not have any neuroimaging data and their findings relating *TNFα* -308 to hemiplegia in very preterm newborns may point to a mechanism of injury involving unilateral vascular causes, rather than cPVL specifically. *TNFα* -308 A allele has previously been reported in association with IVH in preterm newborns (1120-1122), although not consistently (1123). In the current study, the children with cPVL and CP also had some degree of hemorrhage, ranging from mild to severe. cPVL and IVH do indeed co-occur, especially in the severe range (1124-1127). With such small numbers, it is not possible with to disentangle the effects of cPVL and IVH, and further insights may be gained by collecting MRI data.

This study did not support previous association between *IL1β* -511 and CP in case-control studies of very preterm newborns with cPVL (908) and at all gestational ages (1028), or the protective effect in preterm newborns (1102). The large South Australian case-control study also reported a protective effect only in non-spastic CP when analysed as additive minor allele effects (162, 917). There was also no evidence supporting an association between CP and *IL6* -174 (see 2.5.7).

2.5.6 IL1β -511 is associated with cognitive skills at 5y

This study produced first evidence of an association between *IL1β* -511 and cognitive skills at pre-school age, measured with the BAS score. The T allele has been previously reported in association with neurodevelopmental delay at 2 years old (Bayley Scales of Infant Development, mental and/or psychomotor development index <85 in the absence of CP) (905). Interestingly, in our study children with the CC genotype scored on average approximately 8 points less compared to children with the CT genotype (95% CI for the mean difference: 2.84-15.02). Evidence was weaker in relation to the rare TT genotype (95% CI for the mean difference: -4.90-15.94) though in the same direction. This apparent incongruence in direction of effect may be due to haplotype effects, which could not be investigated in the current study due to lack of genetic data on the surrounding genetic sequence (see 5.1.1).

The mean score falls within the normal range of the BAS score, which is within 1 standard deviation from the population mean (85-115), with children below 3 standard deviations (<55) requiring special needs education and children with intermediate scores having variable requirements (1065). Notably, randomised controlled trials (RCTs) of neuroprotective strategies have previously proposed clinically meaningful minimum differences in cognitive scores ranging from half (i.e. 7.5 points) to a third (i.e. 5 points) of a standard deviation (276, 1128, 1129).

In the sensitivity analyses, a suggestive association emerged specifically with the non-verbal reasoning subscale. The subscales reflect the three main processing information systems in the Horn-Cattell model of structural intelligence, i.e. fluid intelligence, crystal intelligence and visuo-spatial intelligence (1130-1132). Fluid non-verbal reasoning is thought to be at the heart of the most complex cognitive processes and involve integration of different processing sub-systems, including visual and auditory (1133, 1134). The structural correlate for fluid reasoning/non-verbal ability is thought to be the frontal lobe (1135, 1136). This is also the last area to complete myelination, well into the second and third decade of life, consistent with the time course of maturation of cognitive functions in childhood and adolescence (101, 1137-1139). Thus, developmental deficits involving the frontal lobe may become even more evident at a later stage and it would be interesting to re-assess the association at school age and during adolescence, as the complexity of cognitive tasks and potential interactions with environmental stressors increases. Additional cognitive outcomes could be assessed including the WISC-IQ (1140-1142) and the NEPSY, which has been previously used in the ELGAN

studies of extreme preterm newborns (606) and in combination with the BAS in studies of mild cognitive impairment in term newbors with encephalopathy (260).

IL1β -511 was not associated with the Griffiths score at 2 years. This is consistent with the known good specificity (accurate prediction of the absence of later impairment) but poor sensitivity (accurate prediction of later impairment) of 2 year outcomes in relation to school age outcomes. Assessment at 2 years is indeed good for predicting the more severe disabilities (e.g. CP, severe cognitive impairment, blindness, deafness), which tend to emerge earlier and remain stable over time (1143). However, predictive power for the milder range of disabilities is modest as developmental trajectories are highly variable, due to increasing exposure to different environmental factors and increasing complexity of cognitive tasks (1082, 1144-1150). In a meta-analysis with over 3,000 very preterm/very low birth weight children, almost half of the children developing some degree of cognitive impairment at school age were classified as having normal development at 2 years (1143).
Future studies should consider the issue of choice of control, since it has been shown that using the normative mean of the psychometric test leads to underestimating cognitive impairment compared to using a selected sample of control children (e.g. classmates or term-born children) (28, 243, 1151).

2.5.7 IL6 -174 may be associated with cPVL at birth

There was suggestive evidence that *IL6* -174 was associated with cPVL. This was the variant most affected by low power in the univariable analyses, since the complete cases was the smallest in the study (n=137). Inclusion of 20 newborns with moderate white matter injury (i.e. persistent periventricular echodensities not evolving into cysts and ventricular dilatation) in the sensitivity analyses strenghtened evidence. Interestingly, the CC genotype has also been found in association with MRI-defined volume loss in the deep grey matter in very preterm newborns (1031). Other studies have reported no association between *IL6* -174 and cPVL (1032, 1152). Similarly, a large study of very low birth weight newborns produced no evidence of an association with brain injury or death (defined as IVH grade IV, PVL, ventricular-peritoneal-shunting or death) (1029). This would support the finding of elevated levels of TNFα and IL1β but not IL6 in post-mortem cPVL brains (579), though this may also be due to poor replication and different cytokine kinetics and temporal profiles during and after injury.

The finding that *IL6* -174 CC genotype may be associated with moderate to severe white matter injury but not with motor and cognitive impairment aligns with the original study using the APIP cohort (1030). On the other hand, a study reported an association with cognitive impairment (Griffith and Kaufmann assessments) in children with cPVL, proposing that rather than causing cPVL, the variant may modify the severity of neurodevelopmental impairment in children with cPVL (1032).

Findings relating *IL6* -174 and CP are also mixed: some studies did not find evidence of association in very preterm (908) and moderately preterm newborns (1032), and the current study did not produce evidence of an association either. On the other hand, associations have been reported with both quadriplegia and hemiplegia at all gestational ages (163, 918, 1027, 1115). In a meta-analysis of 11 case-control studies with 17 SNPs previously implicated in CP, the C allele of *IL*-6 -174 was the only surviving association (912). A large South Australian case-control study reported that having one or two copies of the minor allele (dominant model) increased risk of CP both in the total sample and in the term subset (162, 917). *IL6* -174 has also been reported in association with neurodevelopmental impairment (Bayley <85) in the absence of CP (905). The inconsistent findings highlight the need for replication in independent samples.

2.5.8 EAAT2 variants are in Hardy-Weinberg disequilibrium

The *EAAT2* gentoypes were found to be in Hardy-Weinberg disequilibrium (HWD), with more heterozygotes than expected based on the observed allele frequencies. Evidence of HWD suggests infringement of one or more HW assumptions due to technical artefacts or biological effects (1153-1155). Genotyping error is usually the main source of HWD, with population genetic causes (non-random mating, non-negligible migration and mutation rates, natural selection) usually playing a minor role in genetic association studies (1156). Genotyping errors due to pyrosequencing seem unlikely due to the general robustness of this methodology (1157) and the positive evidence of complete concordance with the 10% of the sample re-sequenced by Sanger sequencing (911). Heterozygote excess may also be due to copy number variation encompassing the variant. A search in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) revealed that *EAAT2* lies within a CNV locus where both gains and losses of DNA segments have been recorded; however, CNVs in this region are extremely rare and have not been found in association with relevant phenotypes (e.g. neurodevelopmental delay, schizophrenia) (1158-1160) (Dr Reese E., Cardiff University, personal communication). SNPs in the primers can also cause HWD via

preferential amplification of one allele over the other (1161). Both PCR primers and the pyrosequencing primer were checked for SNPs using the UCSC BLAT alignment tool (dbSNP build 153) and several SNPs were found which were catalogued in the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/) after publication of the study from Rajatileka and colleagues. The majority were very rare (minor allele frequency <0.0001), except for one variant in the pyrosequencing primer common in the African population (7%) but not found in Europeans, which represent the majority of this cohort. Therefore, there is no convinving evidence that HWD was due to SNPs in the primers.

A possible explanation for HWD is selection bias for admission to the study (1162). Genotypes may have been systematically selected into the study because of unmeasured factors affecting inclusion or assessment, such as gestational age or demographics of the hospital. There is also a small chance that HWD is caused by an actual biological effect driven by natural selection, i.e. the *EAAT2* genotypes may confer a selective advantage/disadvantage, affecting survival/mortality and therefore selection into the study. Indeed, the preterm newborns with the most severe brain injuries who died in the first days of life were not included in this study of a developmental intervention. Therefore, if the homozygous genotypes conferred a selective disadvantage, they could be missing from the cohort due to early death; if the heterozygote genotypes conferred a selective advantage, they could be overrepresented in the cohort. It is not possible to disentangle these effects without a prospective study collecting genotypes during pregnancy, so that data from stillbirths and early neonatal deaths is included. This may be achieved by extracting cell free fetal DNA (cffDNA) emanating from the placenta and circulating in maternal blood from as early as 5 weeks of gestation (1163-1165). This method has been widely studied in the context of non-invasive prenatal diagnosis of aneuploidies (1166-1168), and has been proposed as a tool for earlier identification of pregnancies at risk for adverse outcomes (e.g. pre-eclampsia and foetal growth restriction) (1169-1174)

Interestingly, the variants were in HWD also in the 1000 Genomes total and British but not CEU samples, but the direction of effect was opposite, with loss of heterozygotes. Common genotyping error sources of loss of heterozygotes include deletions, multi-allelic SNPs, and SNPs in the primers. It is difficult to make direct comparisons since the genotyping techniques used in the 1000 Genomes were different. However, the magnitude of the deviation was around 7% in both samples, with the level of evidence being relatively low in our sample, thus it remains to be seen whether these effects have any biological relevance.

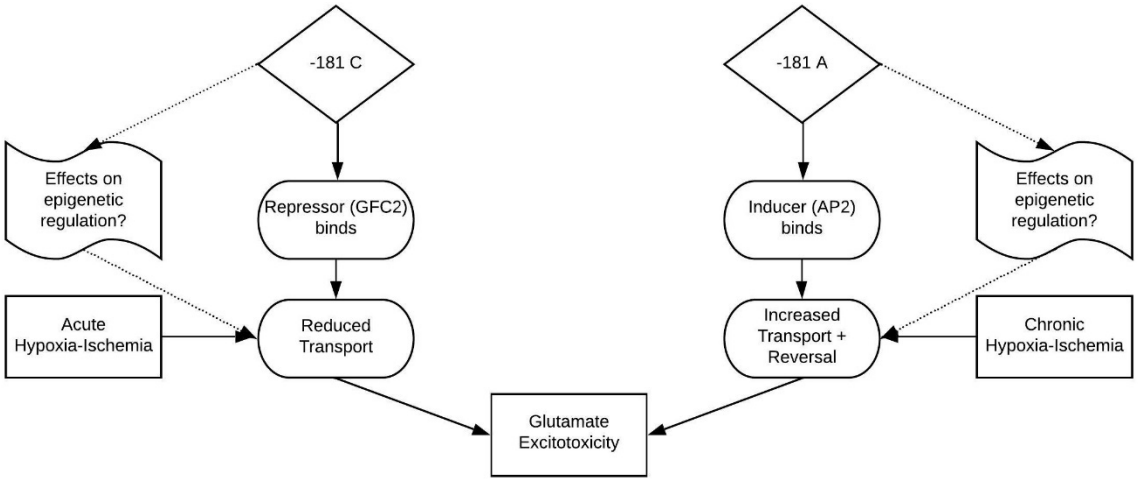
2.5.9 EAAT2 variants remain suitable candidates for future studies

Sensitivity analyses produced suggestive evidence of an association between the glutamate transporter variants and the verbal BAS subscale. Verbal skills at school age have been reported in association with the volume of the corpus callosum (1175). EAAT2 is dysregulated in the white matter lesions of cPVL brains (775), which may affect volume of the corpus callosum. It can be hypothesised that genetic influences on EAAT2 dysregulation may contribute to interindividual differences in vulnerability of white matter structures and related long-term functions. While the evidence remains suggestive in this study, the potential role of *EAAT2* variants in long-term cognition and verbal skills certainly remains worth exploring in larger cohorts. This is of particular interest, since a GWAS of educational attainment with over 100,000 individuals in the discovery sample and 25,000 in an independent replication sample identified 3 non-coding SNPs near 4 genes involved in NMDA glutamatergic signalling (*KNCMA1*, *NRXN1*, *POU2F3*, and *SCRT*) (1176, 1177).

The *EAAT2* variants remain good candidates for future genetic association studies, due to their large effect on gene expression *in vitro*. A construct with 2 copies of the minor allele at both *EAAT2* variants (-200 AA/-181 CC, i.e. 2 A alleles at the genotype combination) showed a staggering 70-80% reduction in basal promoter activity compared to a construct with the reference genotypes (-200 CC/-181 AA) in fetal rat astrocytes (911). The increasing number of A alleles at the *EAAT2* variants has also been reported in association with fMRI-defined reduction in cortical connectivity (1178). It is not known whether the variants affect transcription via epigenetic effects (e.g. DNA methylation). Unfortunately, it is not straightforward to assess mQTL effects for these variants, since they belong to a list of flagged SNPs that Illumina advises to discard from any analyses due to technical limitations of their arrays, with type I probes hybridising poorly and unpredictably to regions containing SNPs (https://support.illumina.com/array/array_kits/infinium_humanmethylation450_beadchip_kit/downloads.html) (1179). On the other hand, the -181 variant has been shown to alter transcription factor binding to the promoter of *EAAT2*, with the minor C allele abolishing the binding site for activator protein 2 (*AP2*) and creating one for the repressor transcription factor GC-binding factor 2 (*GCF2*) (1046). Therefore, preterm newborns with the major A allele may be less able to dynamically suppress *EAAT2*, which may be detrimental if transport activity becomes impaired, reverse transport is established and *EAAT2* becomes a source of extracellular glutamate (668, 751, 753). To this end, these variants may be good

candidates for studies in growth-restricted preterm newborns exposed to chronic intrauterine hypoxia, where being unable to dynamically suppress *EAAT2* may be detrimental if reverse transport is established (1180, 1181). On the other hand, having the minor C allele may be more detrimental following acute HI insults, when glutamate transport is lost in the acute phases (Figure 2.7).

Figure 2.7. Proposed model for differential effects of *EAAT2* genetic variants in acute and chronic hypoxia-ischaemia

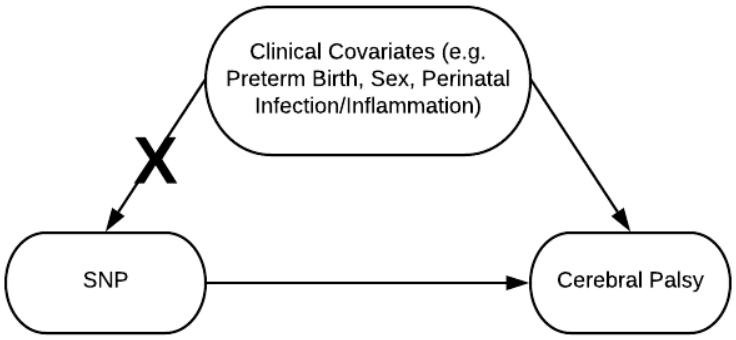


2.5.10 Considerations on SNP-outcome and SNP-clinical covariate-outcome associations

The existence of linkage disequilibrium (LD) patterns in the human genome affects the interpretation of all genetic association studies. Any variant identified here may be causal or simply in LD with a non-genotyped causal variant (912). Moreover, the confounding effect of population stratification must be considered since it can lead to spurious associations and inflated estimates (1182). In this sample, non-Caucasian children were more likely to have discordant genotypes (1 or 3 A alleles) at the *EAAT2* -200/-181 genotype combination. Population stratification bias may occur if ethnicity was also associated with the relevant outcome via mechanisms independent of the genetic effect. On one hand, larger studies sampling from different populations are required to assess whether discordant genotype combinations are indeed more common in non-Caucasian ethnicities. On the other hand, experimental studies more comprehensively assessing the effect of 0, 1, 3 and 4 A alleles on *EAAT2* expression *in vitro* (e.g. via site-directed mutagenesis) may offer more insight into the functional path between genotype and phenotype (1183).

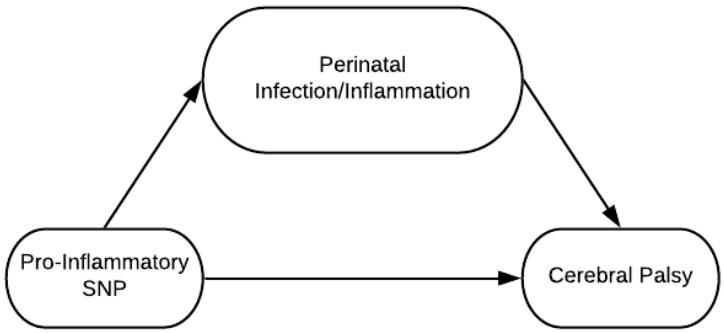
Due to the low power issue, multivariable analyses including the clinical covariates could not be carried out. Published candidate SNP studies have previously adjusted genetic effects on CP by a range of perinatal factors, including these and others (e.g. maternal age, smoking) (917). However, from an epidemiological perspective none of these clinical covariates can be a confounder for these genetic effects because genetic variants are inherited randomly at meiosis (1184). Confounding may occur for example with genetic variants located on sex chromosomes and their effect on outcomes independently associated with sex. However, clinical covariates such as perinatal inflammation or gestational age cannot influence which autosomal variants are inherited by the foetus (Figure 2.8), making adjustment unnecessary (1184).

Figure 2.8. Directed acyclic graph showing that clinical covariates cannot confound genetic effects on neurodevelopmental outcomes



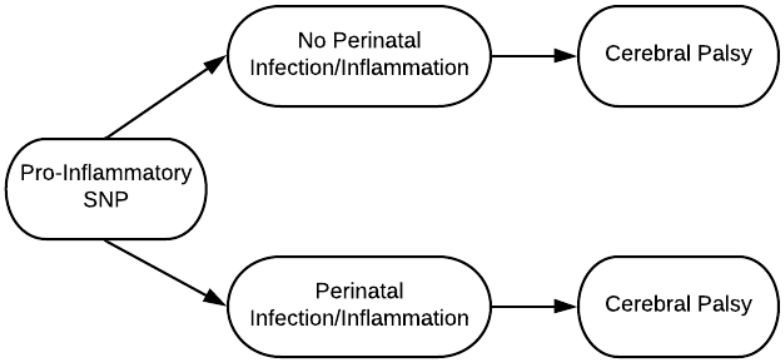
On the other hand, clinical covariates could be analysed as mediators, interactions/effect modifiers via regression modelling in future larger studies. These concepts are interrelated but different, and the choice of the analytical framework will depend on the specific research question. For example, perinatal inflammatory exposures may lie on the causal pathway between these SNPs and neurodevelopmental outcomes as mediators (Figure 2.9). *IL6* -174 has been reported in association with histological chorioamnionitis and neonatal infection in preterm newborns (1098, 1185-1187), and high levels of IL6 have been found in histological chorioamnionitis (628, 630). Similarly, the genetic variants may contribute to neurodevelopmental impairment also by directly affecting risk of preterm birth (1188).

Figure 2.9. Directed acyclic graph of the SNP-cerebral palsy association with clinical covariates as mediators



In gene × environment interaction or effect modification analyses, no causal assumptions are made, instead the rationale is that genetic effects may vary with the covariate, e.g. newborns exposed to perinatal stresses (e.g. infection/inflammation) vs to those who are not. Effect modification analyses differ subtly from interaction analyses in that the focus is specifically on the genetic effect and whether it differs with the covariate (e.g. biological sex) (1189).

Figure 2.10. Directed acyclic graph of the SNP-cerebral palsy association with clinical covariates as effect modifiers



If clinical covariates are neither confounders nor mediators nor effect modifiers but are known *a priori* to affect the outcome, adjusting has been proposed as a way to increase power by reducing residual variance in the outcome not due to the exposures under study (1190). This adjustment can only be done if the SNPs are not associated with the covariate, otherwise bias will be introduced inducing a spurious association between SNPs and outcome (1191). Maternal age (both young and advanced), maternal education and socioeconomic status are known risk factors for CP. Although this data was not available for the APIP cohort, future studies should aim to include this information for more complex modelling.

2.5.11 Strengths and limitations

This is one of the largest prospective birth cohorts where genetic associations with CP can be investigated in very preterm survivors. The target population is clinically relevant as very preterm newborns represent 85% of surviving very preterm infants and two thirds of preterm children with CP (33). The cohort design bypasses the issue of choice of suitable controls (e.g. all healthy newborns vs matched by gestational age) in case-control studies (162). Moreover, the study has the advantage of including secondary cognitive and motor outcomes at 2 and 5 years, assessed through standardised assessments well-established in the UK and used worldwide. Indeed, many children with CP suffer from multiple disabilities and motor and cognitive impairment can occur in addition to or in the absence of CP (146, 192).

The greatest limitation of this exploratory study is represented by low power and the related possibility of false negatives and inflated estimates. The power issue was especially severe for the multivariable SNP-SNP interaction analyses, for univariable analyses for SNPs with low minor allele frequencies (e.g. *IL1β* -511), for the rare binary outcomes (i.e. CP and cPVL) and for the 5 year outcomes subject to loss-to-follow up (i.e. BAS, M-ABC). The wide confidence intervals reflect the limited precision in effect estimates with limited sample size. With regard to the reported associations, we are unable to empirically distinguish between association (correlation) and causation. Moreover, the high number of multiple comparison rises the possibility that these may be false positives. All these limitations are best addressed by replication in independent clinically and ethnically homogeneous samples.

Another important limitation of this study is the lack of direct quantification of circulating cytokine levels. Serial measurements from blood spanning the first two postnatal weeks would be ideal, since it has been shown that in extreme preterm newborns persistently high levels of cytokines over multiple days (i.e. sustained inflammation) have better predictive power for neurodevelopmental impairment than single measurements, and measurements at day 7 and especially day 14 have better predictive power than day 3 (604, 611, 612). Additionally, neuroimaging evidence was limited to cranial ultrasound, which has modest sensitivity for white matter injury. Ultrasound scans are likely to have missed less severe non-cystic forms of PVL, which can only be detected through more sensitive MRI scanning (113). Importantly, disorganisation of white matter tracts can affect white matter function and neurodevelopment in the absence of anatomical lesions and this can only be studied with more advanced structural (e.g. tractography) and functional connectivity MRI techniques (632).

2.6 Conclusion

In conclusion, subject to replication, the current study supports evidence that common regulatory genetic variants thought to influence circulating levels of key pro-inflammatory cytokines *TNFα* and *IL1β* contribute to risk of neurodevelopmental impairment in children born very preterm. This study replicated previous evidence of association between *TNFα* -308, cPVL and CP in children born very preterm, and produced first evidence of an association between *IL1β* -511 and cognitive skills at 5 years of age. It also produced suggestive evidence of an association between *IL6* -174 and moderate to severe white matter injury at birth, and between *EAAT2* variants and the verbal skills cognitive subdomain at 5 years of age. More broadly, the finding supports the role of inflammation as a key pathway of neurodevelopmental impairment, as well as the role of genetic make-up as a contributor to risk.

3 Transcriptional regulation of key pro-inflammatory cytokines and the glutamate transporter in a rat model of hypoxic-ischemic brain injury at term

3.1 Introduction

Despite evidence of glutamate excitotoxicity as a key pathway to injury after acute HI, regulation of the glutamate transporter in HIE has largely been overlooked. More evidence is available supporting an altered cytokine response following HI, however the mechanisms of such changes in regulation are not known (see 1.5.3). While the consequences of *in utero* hypoxia on reprogramming of the foetal brain epigenome are starting to emerge (see 1.5.5), no studies have yet assessed the effects of an acute HI insult on the epigenome of the term brain. DNA methylation provides a suitable candidate mechanism due to its key biological roles for brain function and development, responsivity to early life environmental stresses and evidence of abnormalities associated with brain disorders (see 1.5.5). Moreover, DNA methylation from blood provides an ideal candidate biomarker of injury with potential clinical use translatable to newborns (see 1.5.5). In this study, the Rice Vannucci rat model of HIE was used to assess whether acute HI alters transcription (this chapter) and promoter DNA methylation (chapter 4) of the main glutamate transporter (*Glt1*) and three pro-inflammatory cytokines (*Tnfα, Il1β*, *Il6*) in two of the most vulnerable brain regions, the cortex and hippocampus, as well as in blood.

The Rice Vannucci rat model is the most established rodent model of HI brain injury in the term-equivalent newborn and has been used for the last 40 years (1192, 1193). The insult involves unilateral carotid artery ligation followed by exposure to a defined period of hypoxia in the P5-P10 (postnatal day 5-10) rat pup, leading to a combined unilateral HI injury which cannot be achieved with either of the insults alone (1193). The contralateral hemisphere exposed to hypoxia but not ischaemia serves as an internal control as it is largely undamaged upon histological examination (1194). In the ipsilateral hemisphere, HI causes neuronal and glial necrosis, microglial activation and astrogliosis, with brain injury typically affecting the cortex and hippocampus, followed by the basal ganglia, subcortical and periventricular white matter (1195, 1196). This reflects typical patterns of brain injury seen in HIE babies, which involve the deep grey matter (basal ganglia, thalamus, and hippocampus), somatosensory cortex, white matter, brain stem and cerebellum (79, 80, 82-88). Importantly, these rats

develop neurobehavioural deficits including gross motor dysfunction and cognitive impairment (e.g. learning and memory), which are largely dependent on the HI parameters (502). Versions of this model and its extension to the P7-P9 mouse have provided valuable insights into the pathophysiology of HI brain injury and the molecular mechanisms involved (1197). One of the major characteristics of this model is the inherent variability in the extent of focal injury observed, so that within a sample of rats receiving the same insult some will have no injury and others will have mild to severe injury (1198, 1199). Our group developed a modified version of the P7 Rice Vannucci model with strict control of both core temperature and HI, which produces around 40% brain tissue loss in the ipsilateral hemisphere relative to the contralateral hemisphere one week after HI (1200). Alongside infarction, this model reproduces the reduction of cerebral blood flow, as well as the impairment of innate postural responses and fine motor dexterity seen in humans (1198, 1200-1203). Importantly, this modified Rice Vannucci model was instrumental in the translational journey of therapeutic hypothermia from bench to bedside (702) and is being used for ongoing neuroprotection research for HIE (1199, 1202). The resemblance of short-term brain injury patterns and long-term motor impairment make this model suitable for evaluation of regulatory changes affecting glutamate transport and inflammation in HIE.

3.2 Hypothesis

The hypotheses of this pilot study are that:

1) acute hypoxia-ischaemia causes changes in mRNA transcription of the main glutamate transporter (*Glt1*) and three key pro-inflammatory cytokines (*Tnfα, Il1β*, *Il6*) in the term-equivalent rat brain within the first 24 hours (this chapter)

2) any observed or latent transcriptional changes in *Glt1* and *Tnfα* in the brain are at least in part mediated by DNA methylation changes in the respective gene promoters (chapter 4)

3) DNA methylation of *Glt1* and *Tnfα* in the brain correlates with DNA methylation in blood, which may be a clinically useful peripheral biomarker of injury (chapter 4)

3.3 Materials and methods

3.3.1 Study design

The experiments up to tissue collection were carried out at the University of Essen, Germany, in accordance with the Animal Research: Reporting of *in vivo* Experiments (ARRIVE)

guidelines with government approval by the State Agency of Nature, Environment and Consumer Protection North Rhine-Westphalia, Germany. Hemmen Sabir and Ela Chakkarapani assigned the pups to the different groups, and carried out anaesthesia/experimental HI, killing and tissue collection, in order to minimise operator-related variability. I observed these procedures and, together with Hemmen Sabir, dissected the brains into regions of interest (left and right cortex including subcortical white matter, hippocampus, thalamus, a region enriched for the corpus callosum, cerebellum/brain stem). Brain and blood samples were transported to Cardiff University for downstream molecular analyses.

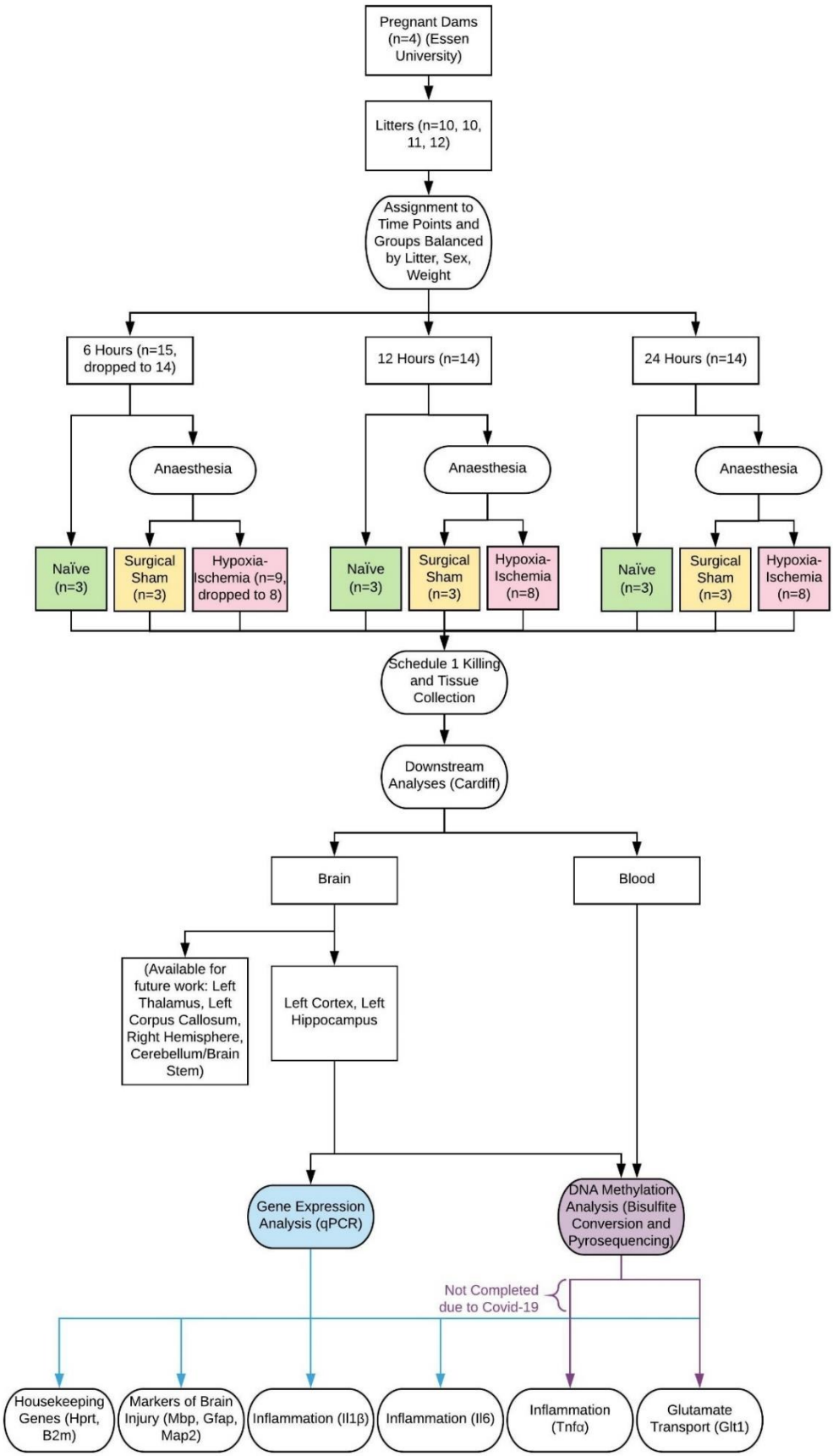
4 litters (n=10, 12, 11, 10) were included, for a total of 43 P7-8 pups. The final sample size was 42, since the "sentinel" rat chosen to carry the rectal temperature probe died at the end of hypoxia. The experimental design included 3 time points (6h, 12h, 24h, n=14 for each time point), each of which featured 3 experimental groups: naïve (n=3), surgical sham (i.e. anaesthesia, n=3) and hypoxia-ischaemia (anaesthesia and surgical ligation of left carotid artery, followed by hypoxia, n=8), with a total of 9 groups (Figure 3.1). The design was therefore unbalanced, with a higher number in the HI group informed by previous experience from our research group and others of high variability and wide distribution of injury in the model.

In terms of genes, mRNA expression was assessed for all candidate genes explored in chapter 1, i.e. the main glutamate transporter in the brain (*Glt1*) and three key pro-inflammatory cytokines (*Tnfα*, *Il1β*, *Il6*). Two housekeeping genes (*Hprt* and *B2m*) with stable expression across all groups were included to normalise gene expression and account for differences in the starting amount of template. *Hprt* is an enzyme catalysing salvage and recycling of purines for building of DNA and RNA. *B2m* is a component of major histocompatibility complex class I molecules. Previous work with this model has shown that the ideal time to assess brain injury (brain volume loss) is 7 days post-HI (1199, 1200, 1202), which was not allowed by the early time points in this pilot study. To support validity of the model, gene expression of established markers of injury was also measured, i.e. *Mbp* for myelin injury, *Map2* for neuronal injury, and *Gfap* for astrocyte activation. Markers of microglial activation (e.g. *Iba1*) and apoptotic cell death (e.g. *caspase 3*) could not be included since this project did not involve histological analyses, which are required to comment on density, morphology, and localisation of these markers.

Promoter DNA methylation analysis focused on *Glt1* and *Tnfα*. Unfortunately, DNA methylation of *Tnfα* could not be assessed due to COVID-19-related disruptions in the final stages of the PhD. The imprinted gene *Peg3* was also included as an internal control for the DNA methylation methodology, based on an expected methylation value of 40-60% (0% on one chromosome and 100% on the other chromosome).

In terms of brain regions, priority was given to analysis of the ipsilateral cortex and hippocampus in the transcriptional analyses (brain: n=84). In the DNA methylation analyses, the ipsilateral cortex was assessed alongside peripheral blood (brain: n=42, blood: n=42).

Figure 3.1. Experimental design of the pilot study



3.3.2 Rice Vannucci rat model of moderate hypoxic-ischemic brain injury at term

3.3.2.1 Preparation and anaesthesia

Dams and litters were cared for in the local in-house animal facility at Essen University. Between experiments, pups were kept in the animal facility with their dams under a 12h:12h dark:light cycle at 21°C room temperature. Dams had access to food and water *ad libitum*.

The evening before the experiment, pups were temporarily separated from their dams, weighed, sexed, and randomly assigned a numerical ID. This was marked on their back, using a different colour marker for each litter. The 42 pups in the final sample belonged to 4 litters (n=10, n=12, n=11, n=9), 24 were male (57%) and mean birth weight was 15 g (±1.26). Pups were subsequently assigned to experimental groups and time points, ensuring balanced representation of litter, sex, and birth weight in each group to minimise confounding. The colour code and number allowed for rapid identification and allocation to treatment on the experimental days (Figure 3.2).

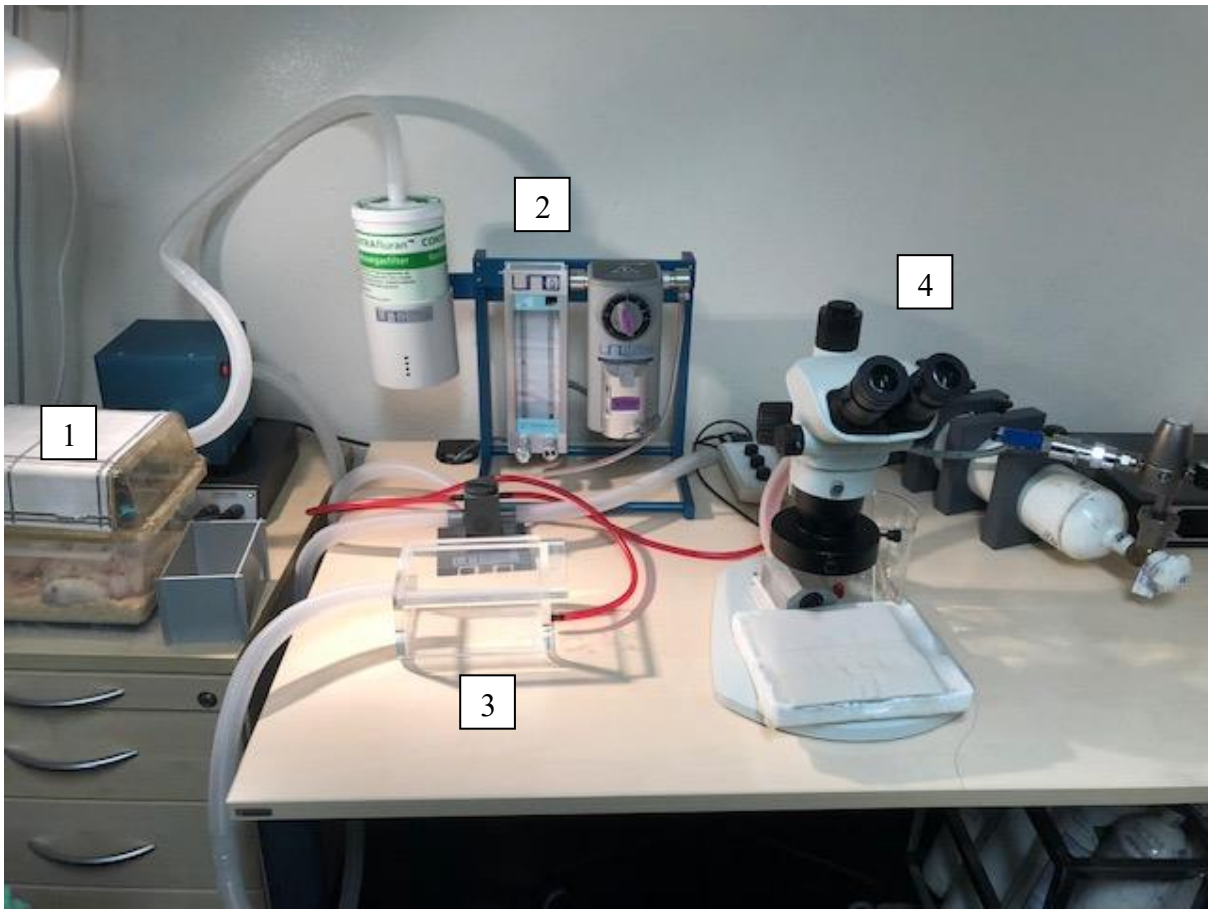
Figure 3.2. Marking of pups with numerical IDs for assignment to experimental groups

On the day of the experiment (P7-8), rat pups from the sham and HI groups were kept in a box on top of a heated mat before and after anaesthesia and experimental HI. The set mat

temperature of 38.5°C was chosen based on previous experiments from our group showing that it keeps rat core temperature stable at 34-35°C (Hemmen Sabir, personal communication). The surgery station, including a dissecting microscope for left carotid ligation, is shown in Figure 3.3.

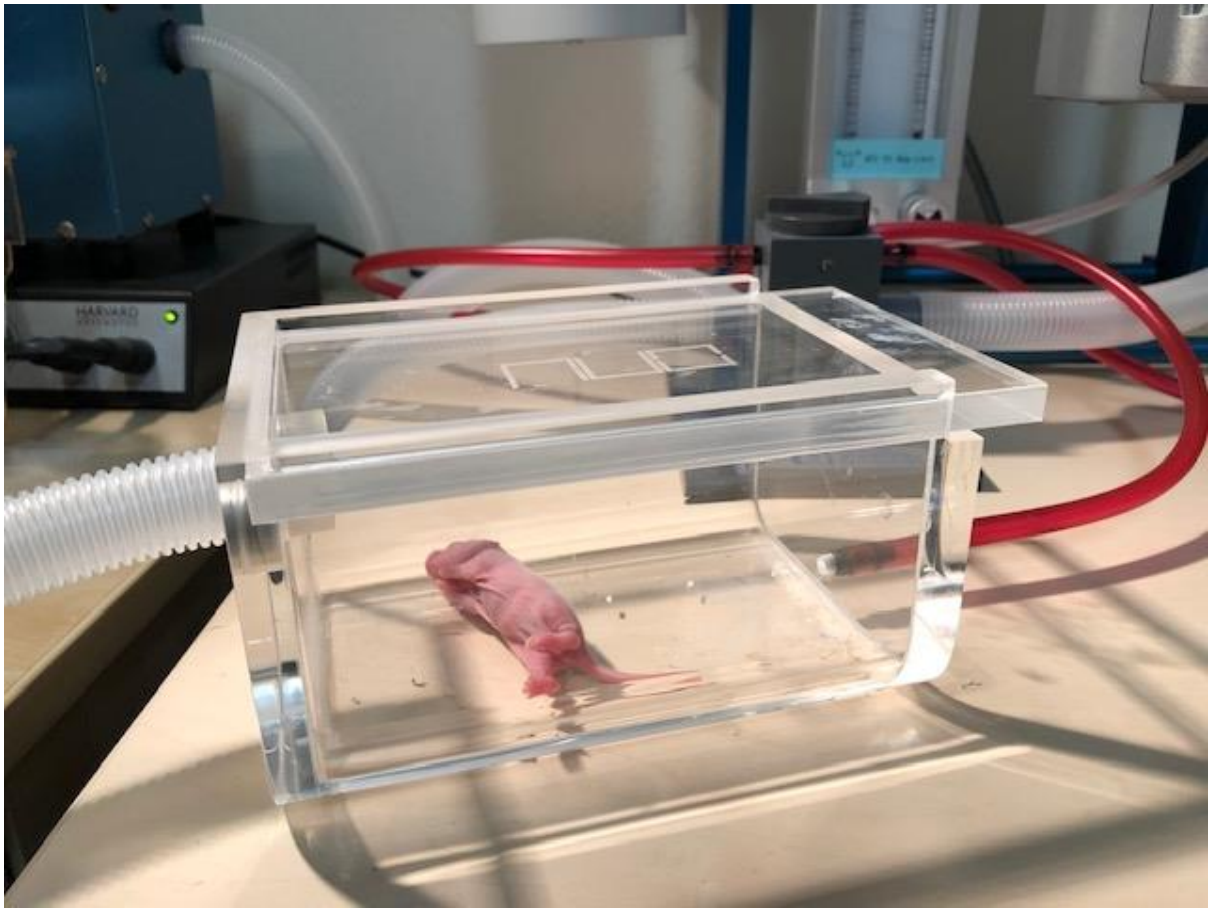
Figure 3.3. Surgery station

Including: 1) box with the rat pups on a heated mat (38.5°C) 2) anaesthesia gases (isoflurane, NO_2, O_2) 3) ventilated anaesthesia chamber 4) dissecting microscope for carotid ligation, also served by the anaesthesia gases. The hypoxia chamber and the CritiCool temperature management system (not included in this picture) were located nearby the surgery station.



Anaesthesia was induced by placing the rat in a ventilated pre-filled anaesthesia chamber with 3% isoflurane in a 2:1 gas mixture of NO_2/O_2, with a gas flow of 1.2 l. The average time for anaesthesia and ligation was 7 minutes (Figure 3.4).

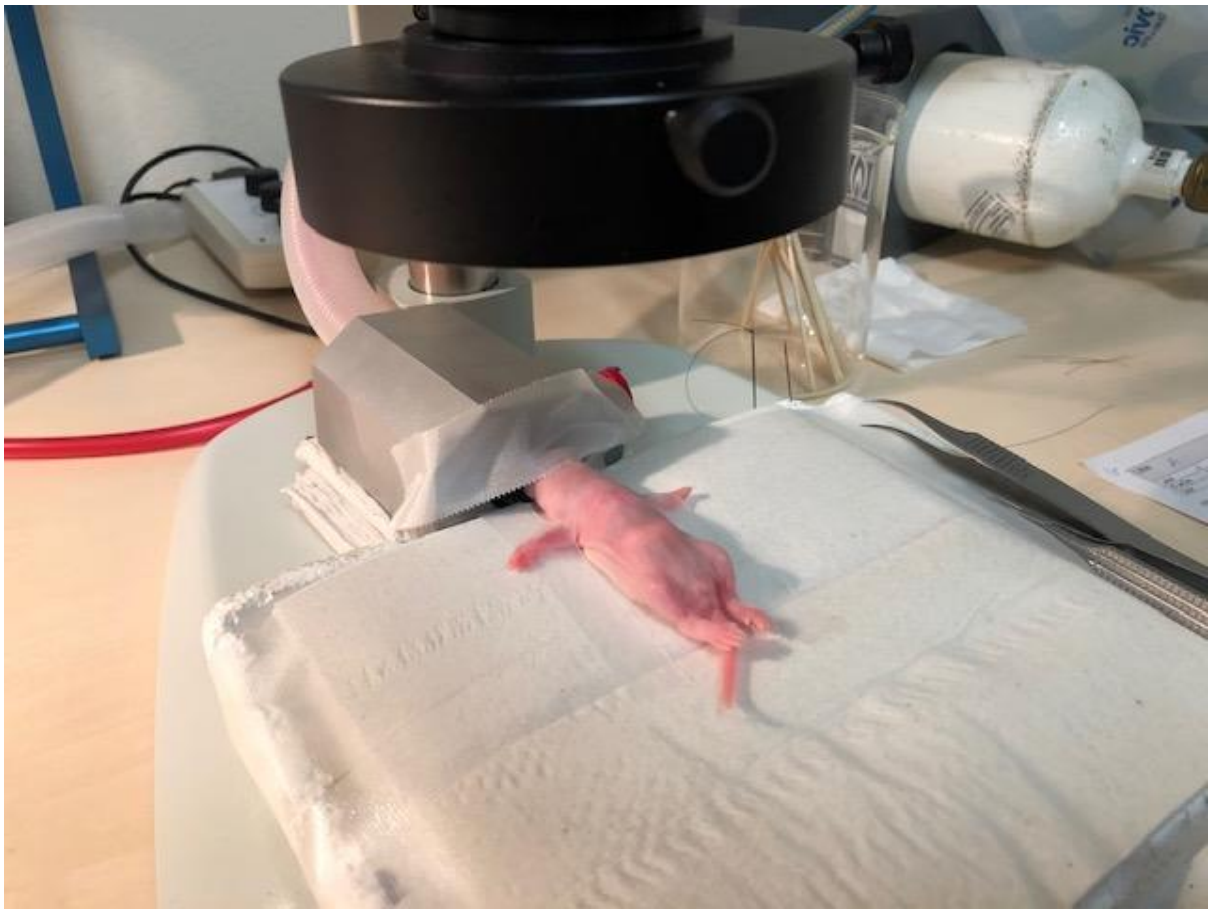
Figure 3.4. Anaesthesia chamber



3.3.2.2 Ischaemia: left carotid artery ligation

After approximately 2 minutes of anaesthesia, the rat was placed under a dissecting microscope and immobilised to the platform underneath with paper tape. The regime of anaesthesia was maintained under the microscope through a face mask (Figure 3.5).

Figure 3.5. Rat pup under the dissecting microscope



Reaching of an appropriate level of anaesthesia was verified by loss of the righting reflex and by pinching a paw. Buprenorphine was injected intracutaneously into the neck. A small incision was made on the left side of the neck. Forceps were used to dissect through the surrounding tissue and the left carotid artery was identified as bright red and pulsating, with a pale muscular wall. The artery was ligated with two sutures of 6-0 surgical silk and cut in the middle. The wound was subsequently closed with surgical silk and the rat was returned to the box on the heating mat with the other pups. After recovery from anaesthesia on the heated mat and regaining of the righting reflex, rat pups were returned to the dams for 30 min recovery period. Each ligation took no longer than 5 minutes. While Hemmen Sabir performed a ligation, Ela Chakkarapani or I placed the next rat in the anaesthesia chamber to ensure the time from ligation to hypoxia was kept within 2 hours.

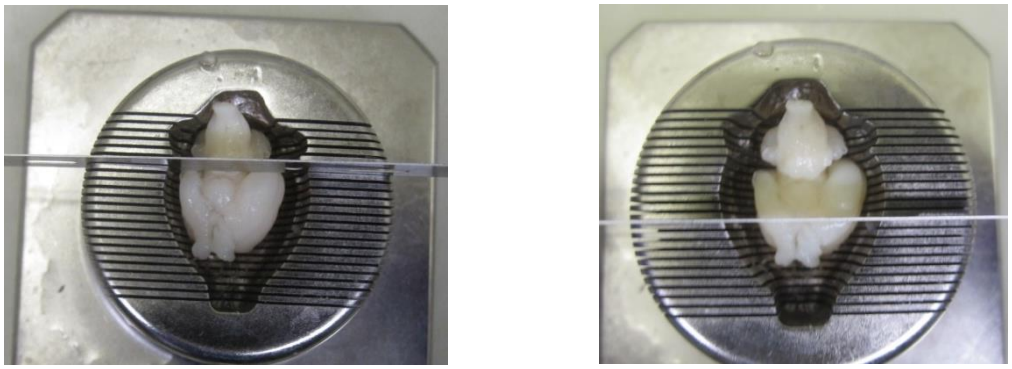
3.3.2.3 Hypoxia

After 30 min recovery with the dam, the rat pups were placed randomly in individual cells within a custom designed metal chamber (1198, 1202). Medical air was delivered in parallel to each cell in the cage. During hypoxia, temperature was continuously monitored by assigning a "sentinel" rat to carry a calibrated rectal temperature probe, secured to the body with paper tape. As the 6 hours HI group included an extra rat compared to the other timepoints, the temperature probe rat was chosen from this group. The cage was placed in a hypoxic chamber on top of a water-filled mat providing constant temperature across all cells. The rectal temperature probe provided continuous measurements to the temperature management system (CritiCool, MTRE), which regulated the temperature of the water-filled mat accordingly to achieve a target of 36°C. This temperature was chosen based on previous studies from our group showing that rectal temperature correlates within 0.1°C with brain temperature and contributes to moderate brain injury (40% area loss in left vs right hemisphere) (702, 1200, 1202). After temperature was stabilised for 15 minutes, the gas supply was set to hypoxia at 8% O_2 (92% N_2). Oxygen concentration was measured continuously and monitored through the Oxycyler system. 8% hypoxia was maintained for 90 minutes or until an animal died. As no animal had died at 90 min, hypoxia was prolonged to 100 min, when the "sentinel" rat carrying the temperature probe died. At the end of hypoxia, gas supply was switched back to room air and temperature raised to 37°C. Rat pups destined for the 12h and 24h time points were returned to the dams in the animal facility, whereas rat pups destined for the 6h timepoint were transferred to the lab facility for killing.

3.3.2.4 Killing and tissue collection

Animals were transported to the lab facilities just before killing and sacrificed to achieve mean time points of 6h, 12h and 24h. Schedule 1 killing was performed by rapid decapitation using surgical scissors. Blood was collected immediately after decapitation using capillary blood collection tubes pre-anticoagulated with K_2EDTA, mixed by gentle inversion, and stored at 4°C. Brains were immediately removed from the skull and placed on a slicer matrix (Zivic Instruments) in a pre-specified orientation (Figure 3.6). The first blade allowed to separate cerebellum and brain stem from the cerebrum. A second blade was inserted 5 grooves rostral from the first one to obtain a single 2.5 mm slice of similar composition across all rats, including portions of the cortex, hippocampus, basal ganglia, and thalamus.

Figure 3.6. Brain on slicer matrix



After separating the cerebellum/brain stem, the slice was transferred to an ice plate and further separated into left (injured) and right (uninjured) hemispheres. From each hemisphere, the following regions were dissected: a small slice (<0.5 mm) immediately proximal to the longitudinal fissure enriched for corpus callosum, followed by cortex (including subcortical white matter), thalamus and hippocampus. In total, 9 regions were collected for each animal, including the cerebellum/brain stem. All dissected regions were placed in pre-labelled tubes and immediately snap frozen in liquid nitrogen. Brain tissue was stored at -80°C and anticoagulated blood was stored at 4°C until the day of travel, then transported on dry ice to Cardiff University for downstream molecular analyses. 3 P7 spleens were subsequently obtained from Hemmen Sabir for optimisation of qPCR (quantitative polymerase chain reaction) cytokine primers since baseline expression is expected to be higher than in brain.

3.3.3 Downstream molecular analyses

Briefly, mRNA and genomic DNA were simultaneously extracted from left cortices and hippocampi, ensuring they originated from the same population of cells. Brain DNA was further processed and analysed alongside blood DNA in DNA methylation analyses (chapter 4). mRNA was cleaned, reverse transcribed to complementary DNA (cDNA) and amplified via qPCR using an intercalating fluorescent dye. Real-time measurements of fluorescence allowed quantification of the transcript in each sample, normalised to endogenous controls (i.e. housekeeping/reference genes). Expression was compared across experimental groups and time points.

3.3.3.1 RNA extraction and clean-up

Genomic DNA and mRNA were simultaneously extracted from each brain sample using Trizol (protocol 1). The extracted mRNA was further cleaned using a column-kit and treated with DNase to avoid genomic DNA contamination (protocol 2).

3.3.3.1.1 Protocol 1: simultaneous DNA/RNA extraction from brain with TRIzol

Precipitation and separation of RNA and DNA

- Transfer samples from -80°C storage to wet ice
- Transfer tissue to FastPrep matrix tubes with lysing beads (MPBio, Matrix D for animal tissues)
- Add 1 ml TRIzol (Invitrogen) per 50-100 mg sample
- Homogenise using FastPrep FP120 homogeniser (Thermo) (3 cycles at 5 m/sec for 15 sec)
- Incubate at room temperature (RT) for 5 min
- Add 0.2 ml chloroform per 1 ml Trizol and shake by inversion for 15 sec
- Incubate at RT for 2-3 min
- Centrifuge at <4000 rpm, 4°C for 15 min with hinge facing outward
- Transfer the colourless upper aqueous phase (containing mRNA) to a fresh tube
- Store remaining sample (interphase containing DNA and organic pink phase containing proteins) at 4°C until DNA extraction

Washing and resuspending of RNA

- Add 0.5 ml isopropanol per 1 ml Trizol and gently mix by inversion
- Incubate at RT for 10 min
- Centrifuge at <4000 rpm, 4°C for 10 min to pellet the RNA
- Quickly remove and discard supernatant
- Add 1 ml 75% ethanol per 1 ml Trizol, mix by pipetting up and down and vortex to resuspend the pellet
- Centrifuge at <4000 rpm, 4°C for 5 min
- Discard the supernatant
- Allow the RNA pellet to air dry (e.g. briefly on heat block with lid open)
- Resuspend in 100 ml nuclease-free water and mix by pipetting up and down
- Incubate at 60°C on thermomixer on gentle shake for 10 min
- Quantify and check purity of RNA on Nanodrop (Nanodrop 8000, Thermo Fisher Scientific) (average at least 2 measurements, use water as blanking buffer)
- Store at -80°C
- Proceed with DNase I treatment (Ambion) and RNeasy MinElute column clean-up (Qiagen)

Washing and Resuspending of DNA

See chapter 4

3.3.3.1.2 Protocol 2: clean-up of RNA (DNase treatment and column clean-up)

- RNeasy MinElute column kit clean-up (Qiagen) (as per manufacturer instructions except for final elution volume)
    - Adjust samples to 100 µl with supplied RNase-free water (i.e. 50 µl RNA + 5 µl DNase buffer + 1 µl DNase = 56 µl: add 44 ul water)
    - Add 350 µl Buffer RLT and mix well by pipetting
    - Add 250 µl 100% ethanol, mix well by pipetting (do not centrifuge and proceed immediately to the next step)
    - Transfer the sample (700 µl) to a spin column and centrifuge at ≥ 8,000 xg (≥ 10,000 rpm) for 15 s
    - Discard the flow-through and place the column in a new 2 ml collection tube (supplied)
    - Add 500 µl Buffer RPE
    - Centrifuge at ≥ 8,000 xg (≥ 10,000 rpm) for 15 s
    - Discard the flow-through and place the column in a new 2 ml collection tube (not supplied)
    - Add 500 µl 80% ethanol
    - Centrifuge at ≥ 8,000 xg (≥ 10,000 rpm) for 2 min
    - Discard the flow-through and place the column in a new 2 ml collection tube (supplied)
    - Write IDs on the side of the tubes, cut lids and keep
    - Centrifuge at full speed for 5 min, with the hinge facing outward
    - Centrifuge at full speed for 5 min, with the hinge facing inward
    - Discard the flow-through and place the column in a new 1.5 ml collection tube (not supplied)
    - Elute in 20 µl in two steps (higher than 14 µl in single elution recommended by manufacturer)
        - Add 10 µl RNase-free water directly to the centre of the membrane and put lid back on
        - Centrifuge at full speed for 1 min and repeat step
- DNase I treatment (Ambion)

- Transfer 50 µl RNA to a fresh tube (if concentration is > 200 µg/ml, dilute RNA to 10 µg/50 µl)
- Add 5 µl DNase I Buffer to the RNA sample (10X to 1X)
- Add 1 µl DNase I (2 U) and incubate at 37° C for 30 min (to remove up to 2 µg DNA)
- Incubate at 75° C for 5 min for heat inactivation of DNase I

- Quantify and check purity of RNA on Nanodrop

3.3.3.2 Reverse transcription of mRNA to cDNA and qPCR

Cleaned mRNA was reverse transcribed to double stranded cDNA (protocol 3), which is more stable than mRNA and can be recognised by DNA polymerase. The RNA to cDNA EcoDry™ Premix (Double Primed) kit (Takara) contains a mixture of oligo(dT) and random primers. Oligo(dTs) are specific for poly(A) tails and allow reverse transcription of full-length mRNAs, random primers are ideal for degraded mRNAs.

Gene expression was measured by quantification of amplified cDNA in each brain sample by real-time qPCR using a fluorescent dye. The following genes were assessed: the four candidate genes (*Glt1*, *Tnfα*, *Il1β*, *Il6*), the three markers of brain injury (*Mbp*, *Gfap* and *Map2*) and two established housekeeping genes (*Hprt* and *B2m*). Inclusion of housekeeping genes with stable expression across groups and time points allows to account for non-biological variation between samples (e.g. amount of input mRNA and PCR reagents, pipetting error, errors in sample quantification) which could otherwise be erroneously attributed to group differences (1204).

qPCR was carried out using the SensiFAST SYBR Green No-ROX kit (Bioline), which includes the SYBR fluorescent dye, DNA polymerase and PCR buffers and enhancers. Each sample was run in triplicate, yielding 126 samples for each of the two brain regions, for each of the 9 genes (total n = 2,268). Three 100-wells qPCR plates/rings (Qiagen) were run for each gene. Each plate was designed to include representatives of all time points and experimental groups. cDNA samples were mixed with the SYBR Green premix and loaded on the rings using a PCR set-up robot (CAS-1200, Corbett) under supervision. The ring was heat sealed and transferred to the Rotor-Gene Q cycler (Qiagen) for qPCR (protocol 4).

During exponential amplification, the fluorescent SYBR Green dye intercalates within the double stranded cDNA. Fluorescence is measured in real time on the Rotor-Gene Q and plotted against the number of cycles by the Rotor-Gene Q software, which automatically

applies quality control procedures (e.g. fluorophore-specific channel filters, removal of background noise). Fluorescence and number of cycles have an exponential relationship or log linear with log fluorescence. The resulting sigmoidal-shape plots (on a linear scale) allow to distinguish a log-linear phase of growth between a baseline phase (i.e. noise level in early cycles) and a plateau phase (i.e. PCR resource exhaustion). A threshold is arbitrarily chosen and set as a horizontal line within the log-linear region of growth of the amplification curve, where precision of the replicates is highest. Fluorescence is quantified as a cycle threshold (Ct), i.e. the cycle at which the amplification curve exceeds the set threshold (1205). A threshold of 0.4 was found to be suitable across all genes and plates. Raw Ct data were obtained in text format from the software for analysis.

Before running the experiments, all qPCR assays were optimised on the Rotor-Gene Q by testing different qPCR primer concentrations with constant temperature and number of cycles. Cytokine primers were optimised in spleen and all remaining primers were optimised in brain tissue. For optimisation, a good quality cDNA sample was run in triplicate with 9 combinations of forward and reverse primer concentrations: 10 µM, 4.3 µM and 0.7 µM (final concentration in qPCR reaction: 700 nM, 300 nM, 50 nM). The combination of primer concentrations was chosen as the one producing the lowest Ct (i.e. earliest detectable change in fluorescence), highest technical replicate reproducibility and minimal noise in the non-template control.

Initially, sample Ct values were found to differ systematically in different plates for the same genes. This was found to be due to a setting on the Rotor-Gene Q software automatically optimising the gain for the green channel at the beginning of quantitation for each plate. Plates were repeated after manually setting the green channel gain value to 8. Moreover, two spare samples were included on all plates as calibrator samples to evaluate consistency in gene expression quantification (1206). Housekeeping genes were quantified separately for each cDNA batch.

3.3.3.2.1 Primers

qPCR primers (Sigma-Aldrich) were designed for the four candidate genes (*Glt1*, *Tnfα*, *Il1β*, *Il6*), the three markers of brain injury (*Mbp*, *Gfap* and *Map2*) and two housekeeping genes (*Hprt* and *B2m*) (Table 3.1), using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). All Primer3 parameters were kept to default settings except for product size (reduced to 80-200 bp) and primer melting temperature (reduced to 58-61°C). Moreover,

primers were forced to span exon boundaries, to ensure selective amplification of mRNA over genomic DNA. Published *Glt1* primers were excluded as, upon inspection, it was found they did not span exon boundaries (1207-1209). Primers for the two housekeeping genes routinely used in mouse by the Cardiff Behavioural Genetics Group (i.e. *Hprt* and *B2m*) were redesigned for the rat genome. All primers were BLASTed on NCBI to check specificity (1210).

Table 3.1. qPCR primers

Primer Name	Primer Sequence	Exon Boundary	Product Size (bp)
glt1_F	CGTGGGCCTCATCATTCATG	1-2	151
glt1_R	GGTGACAGGCAAAGTTCCAG		
tnfα_F	CCACCACGCTCTTCTGTCTA	1-2	90
tnfα_R	TGGAACTGATGAGAGGGAGC		
il1β _F	GGGATGATGACGACCTGCTA	5-6	192
il1β_R	TGTCGTTGCTTGTCTCTCCT		
il6_F	CTGCTCTGGTCTTCTGGAGT	4-5	180
il6_R	AGAGCATTGGAAGTTGGGGT		
mbp_F	CGCAGAGGACCCAAGATGAA	3-4	195
mbp_R	CAGGATTCGGGAAGGCTGA		
gfap_F	TAAGCTAGCCCTGGACATCG	2-3	105
gfap_R	CGGATCTGGAGGTTGGAGAA		
map2_F	TGAACAAGAGAAGGAGGCCC	3-4	174
map2_R	TGTTTAAACGCACTGGGAGC		
hprt_F	AGGACCTCTCGAAGTGTTGG	7-9	137
hprt_R	CCACTTTCGCTGATGACACA		
b2m_F	TCTGGTGCTTGTCTCTCTGG	1-2	126
b2m_R	TGGAACTGAGACACGTAGCA		

3.3.3.2.2 Protocol 3: cDNA Synthesis from mRNA

- Transfer 1 µg mRNA to a fresh tube (measured on Nanodrop)
- Make up to 20 µl with nuclease-free water
- Add mRNA to the premix (RNA to cDNA EcoDry™ Premix (Double Primed) kit, Takara) and mix by pipetting up and down
- Incubate on thermal cycler (Bio-Rad)
    - 42°C for 60 min
    - 70°C for 10 min
- Make up to 500 µl with nuclease-free water

- Store at -20°C
- Proceed to qPCR

3.3.3.2.3 Protocol 4: qPCR

- Load PCR set-up robot (Corbett) with the total volume required for all cDNA samples and non-template controls (NTC) (Table 3.2)

Table 3.2. qPCR recipe

Reagent	Volume (1 reaction)
SensiFAST SYBR Green	12.5 µl
Forward Primer	1.75 µl
Reverse Primer	1.75 µl
Nuclease-Free Water	4 µl
cDNA Template/NTC	5 µl
Total	25 µl

- Heat seal plate
- Load ring on RotorGene-Q and run qPCR (Table 3.3):

Table 3.3. qPCR protocol

PCR Cycle Step	Temperature	Time	No. Cycles
Taq Heat Activation	95°C	10 min	
Denaturation	95°C	20 sec	
Annealing	60°C	20 sec	x 40
Extension	72°C	20 sec	

3.3.4 Statistical analysis

The exposures in this study are experimental group (naïve, sham, hypoxia-ischaemia) and time (6h, 12h, 24h), while the outcome is normalised gene expression on the log scale (untransformed ΔCt values). At the analysis stage, the $2^{-\Delta\Delta CT}$ method was used (1211), with the following calculations:

1) Mean sample Ct values obtained by averaging the three technical replicates for each sample. Batch differences were assessed by comparing mean sample Ct values by plate with a one-way ANOVA and plates were repeated where needed

   Sample Ct = arithmetic mean of the Ct values for the 3 technical replicates

2) Untransformed sample ΔCt values (used for statistical analyses and plots), i.e. the log of gene expression normalised to the geometric mean of the housekeeping genes. The geometric mean controls better than the arithmetic mean for possible outliers and differences in abundance:

   Sample ΔCt = Ct(geometric mean of *Hprt* and *B2m*) – Ct(gene of interest)

   This was adapted from the original $2^{-\Delta\Delta CT}$ method, whereby the subtraction was performed as (Ct(gene of interest) – Ct(reference gene)) leading to higher values on the plots indicating lower quantities relative to the housekeeping genes and vice versa; this adaptation does not affect the values but allows easier interpretation of the direction of effect on the plots

3) Untransformed mean group ΔCt values, obtained by averaging the biological replicates for each group (e.g. 6N, 12S, 24HI). A standard error of the mean was also calculated as (SD/SQRT(N))

   Group ΔCt = arithmetic mean of the sample ΔCt values for each of the 9 group/time point combinations

4) Transformed $2^{\Delta\Delta Ct}$ values (used for plots), i.e. fold-change in gene expression for each experimental group in relation its age-matched control group (i.e. naïve group at the same time point). This was achieved first by calculating untransformed ΔΔCt values:

   ΔΔCt = group ΔCt – control group ΔCt (e.g. 12HI ΔCt – 12N ΔCt)

   Back-transformation of logarithmic values ($2^{\Delta\Delta Ct}$) allowed expression as fold-change for each group relative to its age-matched control (which takes a constant value of 1 following subtraction to itself and transformation). The upper and lower limits of the standard error of the mean were calculated by adding and subtracting the standard error of the mean group ΔCt from the ΔΔCt.

Gene expression data are often plotted as fold change ($2^{\Delta\Delta Ct}$), since untransformed ΔCt values on the logarithmic scale are not necessarily intuitive for visualisation. However, it is useful to

plot raw data on the same scale as used for the analysis; moreover, fold-change can be more easily interpreted in treatment vs control designs than in 3 x 3 designs with a different control group for each time point. For these reasons, side-by-side plots were reported of both the untransformed ΔCt (on the logarithmic scale, scatterplots with means and standard errors) and the $2^{\Delta\Delta Ct}$ fold change in relation to respective age-matched controls (on the linear scale, bar charts with potentiated means, asymmetric standard error bars and age-matched controls set as y=1).

A two-way 3 x 3 factorial ANOVA was run separately on 42 left cortices and hippocampi to compare the main effects and interaction of experimental group (naïve, sham, hypoxia-ischaemia) and time (6h, 12h, 24h) on normalised gene expression, i.e. untransformed ΔCt values, which are expected to have a normal distribution (1212). The Scheffe's test was used for *post hoc* analyses to identify which pairs of means were significantly different. While affording less power than the Tukey's and Fisher's tests, it allows comparison of all possible pairs of three means as well as different number of observations per group. Effect sizes were expressed in terms of population (ω^2, omega squared) rather than sample (η^2, eta squared) effect sizes, as, though always smaller, they have been found to carry less sampling error bias in unbalanced designs with multiple groups (1213).

Secondary analyses were planned to partially address the issue of limited power in this small pilot study. Differences were tested between naïve (n=3 per time point) and sham (n=3 per time point). If no differences were detected, the naïve (N) and sham (S) groups were collapsed into a single larger control group (n=6 per time point) and the ANOVA repeated in a 2 x 3 design (N/S vs HI). This type of analysis remains suggestive in terms of evidence level. Sensitivity analyses were carried out for the cytokines and *Gfap* based on the observed non-significant trends for interaction effects between group and time. These secondary analyses merged time points as well as groups in a 2 x 2 ANOVA design based on observations from the primary analyses.

Stringent multiple comparison correction for multiple independent tests (e.g. Bonferroni correction) may not be appropriate in exploratory pilot studies with limited power, whereby the cost of false negatives is potentially higher than that of false positives (1116). Moreover, some degree of correlation may be expected amongst genes that are co-regulated within the inflammatory and glutamatergic pathways and may therefore not represent independent tests. For this reason, uncorrected p-values were reported. The Benjamini Hochberg False

Discovery Rate (FDR) was applied as an alternative strategy for multiple comparison correction, since it does not assume independency of the tests and is less penalising in terms of power by setting an acceptable level of false discoveries (1214). An FDR of 7% was chosen, and it translates in accepting that 1 gene in one of the two brain regions may be a false positive (7% of 14 (n=7 genes per brain region) = 1). To apply the Benjamini Hochberg FDR correction, p-values were ranked from smallest to largest; a critical value was calculated for each p-value as (i/m)Q (i = rank; m = total number of tests; Q = chosen FDR); the largest p-value that was smaller than its Benjamini-Hochberg critical value was considered as significant after correction, alongside all of the p-values higher up in rank. The most suitable way to address the issue of false positives is to interpret results with caution until replication in larger experiments.

To explore co-regulation between genes, Pearson's correlation coefficients were derived inferring the linear relationship between expression of each pair of genes. The coefficients with significant correlations ($p \leq 0.05$) were annotated on pairwise scatterplots without adjustment for multiple comparison. In sensitivity analyses, rats were ranked based on the normalised gene expression values (untransformed ΔCt) for each gene taking into consideration the expected direction of effect based on observed data from this study as well as the literature, e.g. ranking from lowest to highest gene expression for genes expected to be suppressed and highest to lowest for genes expected to be upregulated. The top 10 pups with the most extreme gene expression values for each gene were nominally compared across genes to make observations of suggestive value.

Calculations from Ct values to $2^{\Delta\Delta Ct}$ values were performed using Excel (Microsoft Corp, version for Microsoft 365). Statistical analyses with sample ΔCt and plots of sample ΔCt and $2^{\Delta\Delta Ct}$ were carried out and designed using Stata 14 (Stata Corp, TX, USA).

3.4 Results

3.4.1 Housekeeping genes

There was no evidence that the Ct values for housekeeping genes *B2m* and *Hprt* were affected by group ($p_{B2m}=0.227$; $p_{Hprt}=0.103$) or time ($p_{B2m}=0.880$; $p_{Hprt}=0.272$).

3.4.2 Cortex

3.4.2.1 Markers of injury

Descriptive statistics are reported in Table 3.4. Results from the primary analyses are reported in Table 3.5. Briefly, there was strong evidence of suppression of *Mbp*, a marker of myelin injury, and upregulation of *Gfap*, a marker of astrocyte activation, in the left cortex in the HI group. This effect was independent of time. There was no evidence of changes in neuronal marker *Map2* with group or time.

Table 3.4. Markers of Injury (Cortex): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time (h)	n	*Mbp*		*Gfap*		*Map2*	
			Mean ΔCt	SD	Mean ΔCt	SD	Mean ΔCt	SD
N	6	3	-0.23	0.38	0.09	0.25	0.62	0.39
	12	3	-0.53	0.64	0.06	0.27	0.53	0.32
	24	3	0.22	0.59	0.09	0.45	0.19	0.41
S	6	3	0.21	0.38	0.28	0.34	0.72	0.62
	12	3	0.27	0.37	0.27	0.08	0.86	0.32
	24	3	0.04	0.18	0.21	0.20	0.53	0.22
HI	6	8	-1.12	0.87	0.80	0.37	0.54	0.32
	12	8	-0.85	0.44	0.88	0.78	0.49	0.39
	24	8	-0.78	0.58	1.73	1.37	0.61	0.29

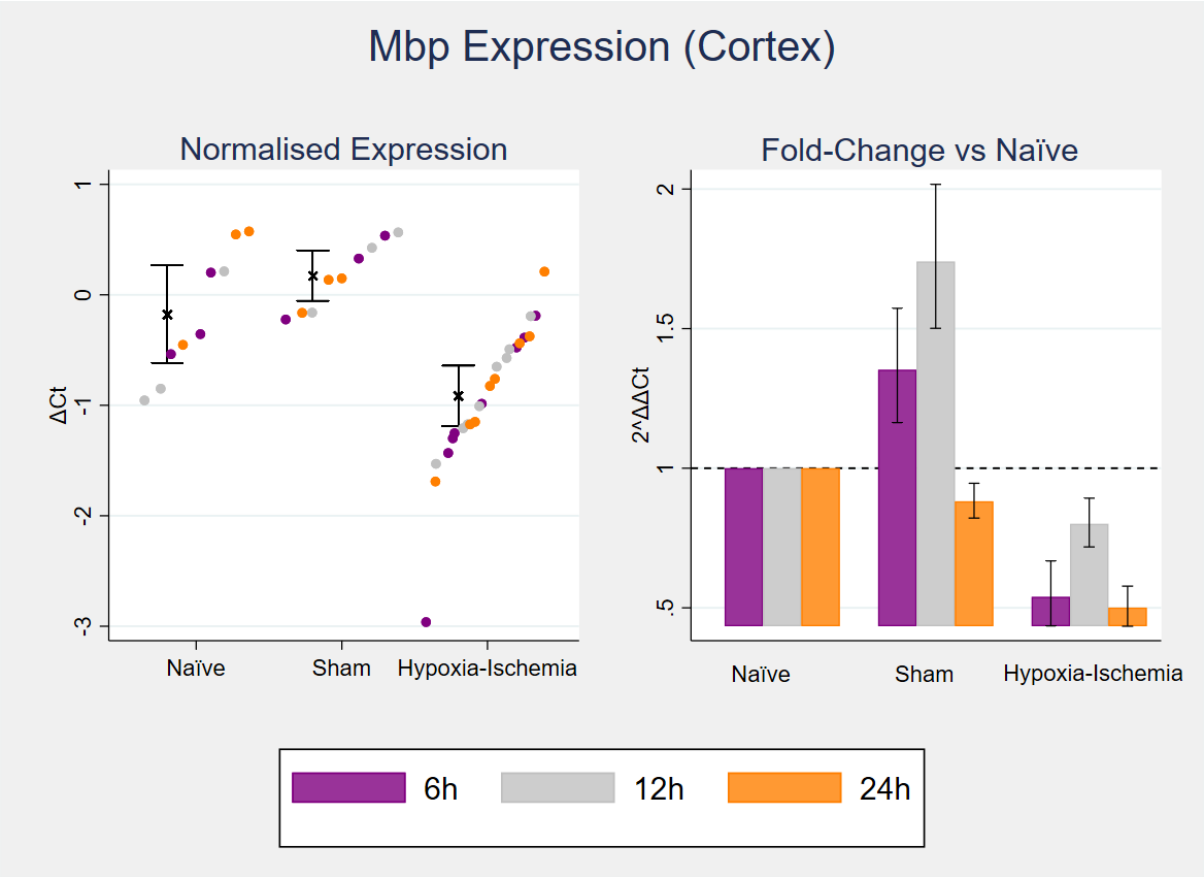
Table 3.5. Markers of injury (cortex): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	p	Effect size	*Post hoc* Scheffe's p
	Group (N vs S vs HI)		2	4.55	13.04	**0.0001**	**0.40**	N (p=0.009) and S (p<0.0001) ≠ HI
Mbp	*Time*	0.61	2	0.16	0.45	0.642	0 (-0.03)	
	*Group*Time*		4	0.25	0.71	0.589	0 (-0.03)	
	Group (N vs S vs HI)		2	4.91	8.42	**0.001**	**0.29**	N (p=0.005) and S (p=0.021) ≠ HI
Gfap	*Time*	<0.0001	2	0.30	0.51	0.603	0 (-0.03)	
	*Group*Time*		4	0.49	0.84	0.513	0 (-0.02)	
	Group (N vs S vs HI)		2	0.15	1.18	0.320	0.01	
Map2	*Time*	0.84	2	0.13	0.97	0.391	0 (-0.002)	
	*Group*Time*		4	0.12	0.89	0.480	0 (-0.01)	

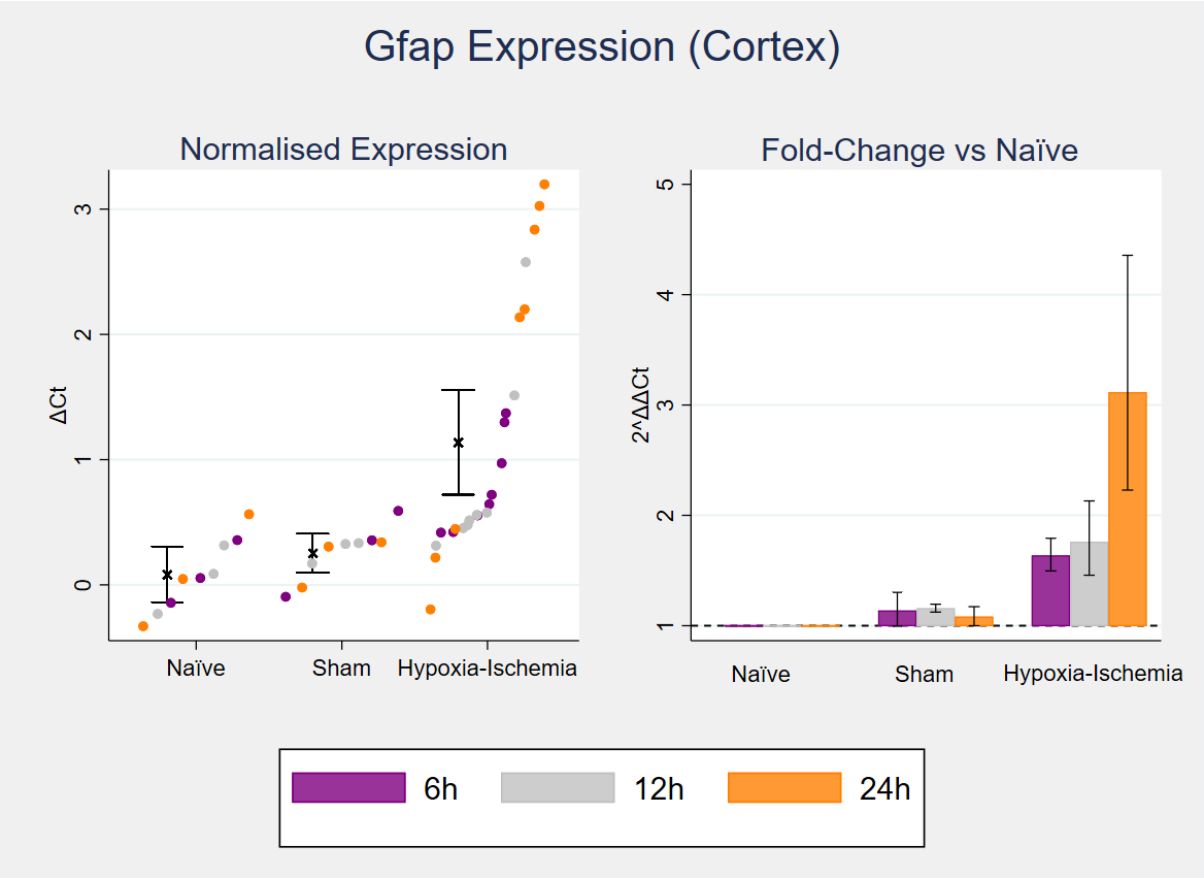
More in detail, there was strong evidence of a group effect on *Mbp* expression (p=0.0001) independently of time, with a large effect size of 40%, i.e. 40% of the variation in *Mbp* expression was accounted for by group (Table 3.5). *Post hoc* analyses revealed that this was driven by the HI group, with no evidence of differences between naïve and sham. The plots of normalised expression and the plot of the fold change reflect this *Mbp* loss in the ipsilateral cortex in the HI group (Figure 3.7). The plot of fold-change shows that expression in the HI groups is 0.5-0.8 fold down (20-50% lower) compared to the age-matched naïve groups at the corresponding time points. In the secondary analyses merging naïve and sham groups, evidence of *Mbp* loss in the HI group strengthened (p<0.00001) and maintained a similar effect size (38%) (Table 3.6, Figure 3.10).

Figure 3.7. *Mbp* expression (cortex): plots



There was also strong evidence of a group effect on *Gfap* expression independent of time (p=0.001), with a large effect size of 29%. As for *Mbp*, this was driven by the HI group, with no differences between naïve and sham in *post hoc* analyses (Table 3.5). Specifically, *Gfap* was upregulated in HI, with a distinct S-shaped bimodal distribution (Figure 3.8). The plot of the fold change shows that expression in the HI groups is 1.6-3.1 fold higher (160-310%) compared to the age-matched naïve groups. Within the HI group, there was a non-significant trend for higher expression at 24h compared to 6h and 12h, with a 3-fold higher expression compared to the naïve group at 24 h. In the secondary analyses merging naïve and sham groups, evidence of *Gfap* upregulation in the HI group strengthened (p=0.0001) and maintained a similar effect size (31%) (Table 3.6, Figure 3.11).

Figure 3.8. *Gfap* expression (cortex): plots



There were no differences in neuronal marker *Map2* in the primary or secondary analyses, and effect sizes were also negligible (Table 3.5, Figure 3.9, Table 3.6, Figure 3.12).

Figure 3.9. *Map2* expression (cortex): plots

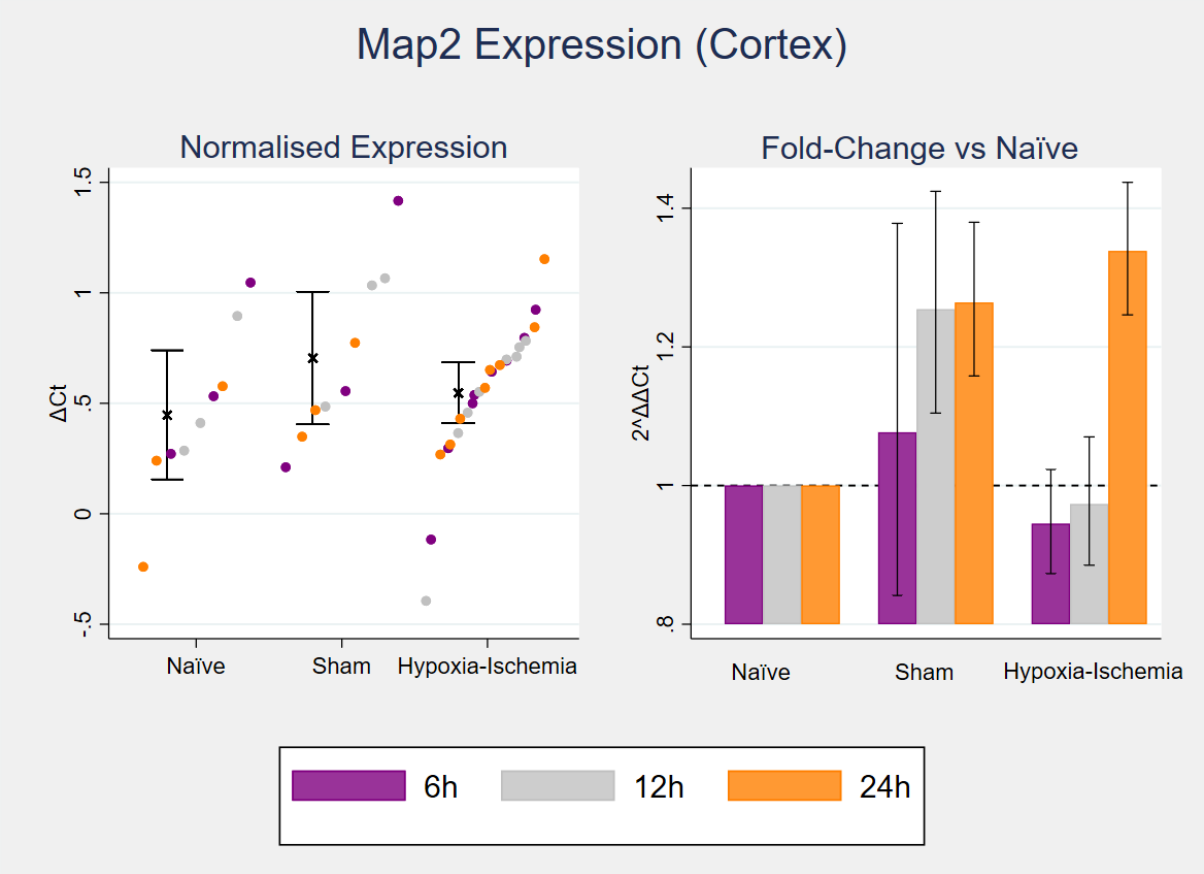


Table 3.6. Markers of injury (cortex): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Mbp	*N vs S*	*Group*	0.16	1	0.55	2.75	0.123	0.11
		Time		2	0.10	0.50	0.617	0 (-0.07)
		*Group*Time*		2	0.37	1.83	0.202	0.10
	N/S vs HI	*Group*	0.56	1	8.56	24.03	**<0.00001**	0.38
		Time		2	0.21	0.59	0.559	0 (-0.02)
		*Group*Time*		2	0.13	0.36	0.698	0 (-0.03)
Gfap	*N vs S*	*Group*	<0.00001	1	0.13	1.59	0.231	0.04
		Time		2	0.02	0.02	0.978	0 (-0.15)
		*Group*Time*		2	0.04	0.05	0.955	0 (-0.15)
	N/S vs HI	*Group*	0.79	1	9.69	18.0	**0.0001**	0.31
		Time		2	0.86	1.60	0.215	0.04
		*Group*Time*		2	0.97	1.80	0.180	0.03
Map2	*N vs S*	*Group*	0.47	1	0.30	1.87	0.196	0.06
		Time		2	0.21	1.31	0.306	0.04
		*Group*Time*		2	0.03	0.16	0.854	0 (-0.13)
	N/S vs HI	*Group*	0.79	1	0.01	0.07	0.799	0 (-0.03)
		Time		2	0.06	0.45	0.643	0 (-0.03)
		*Group*Time*		2	0.21	1.60	0.216	0.03

Figure 3.10. *Mbp* expression (cortex): plot of secondary analyses

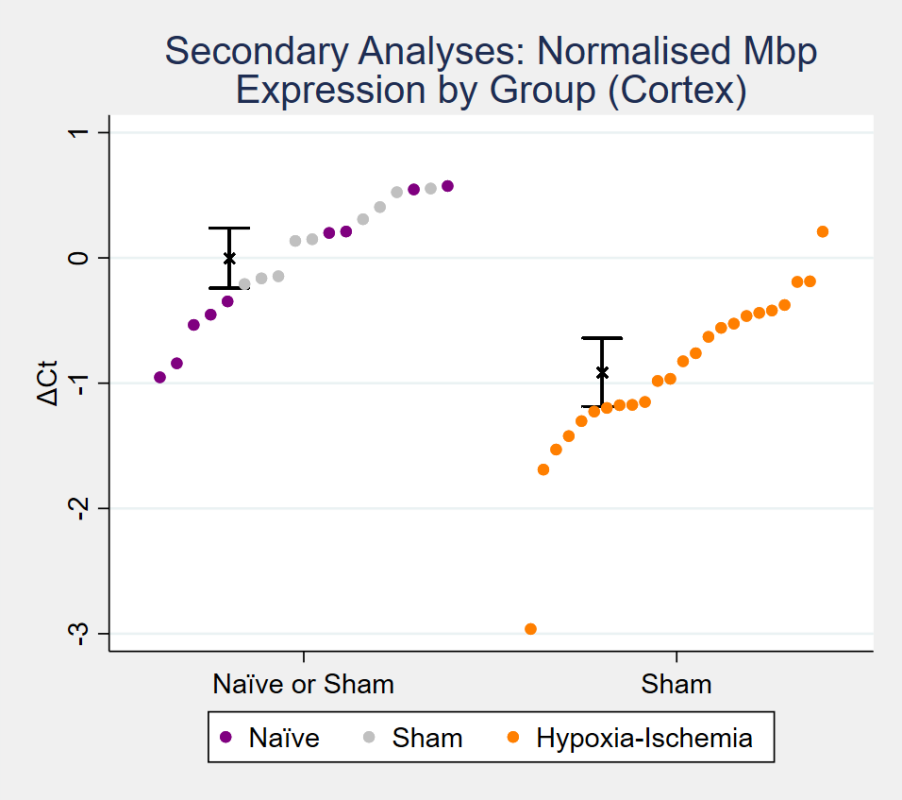


Figure 3.11. *Gfap* expression (cortex): plot of secondary analyses

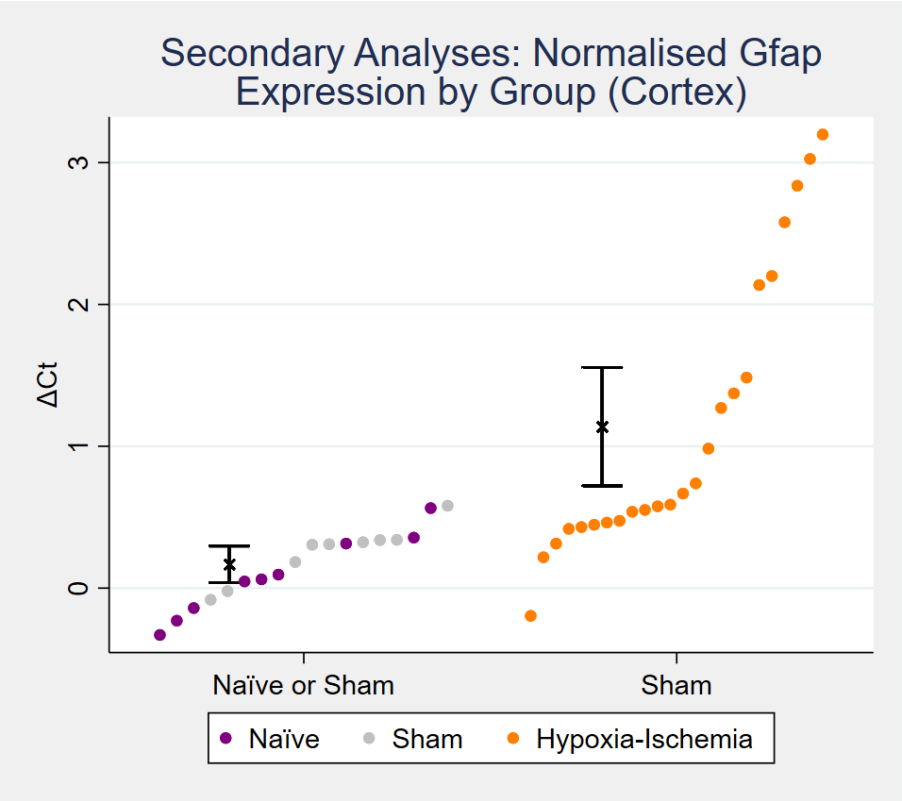
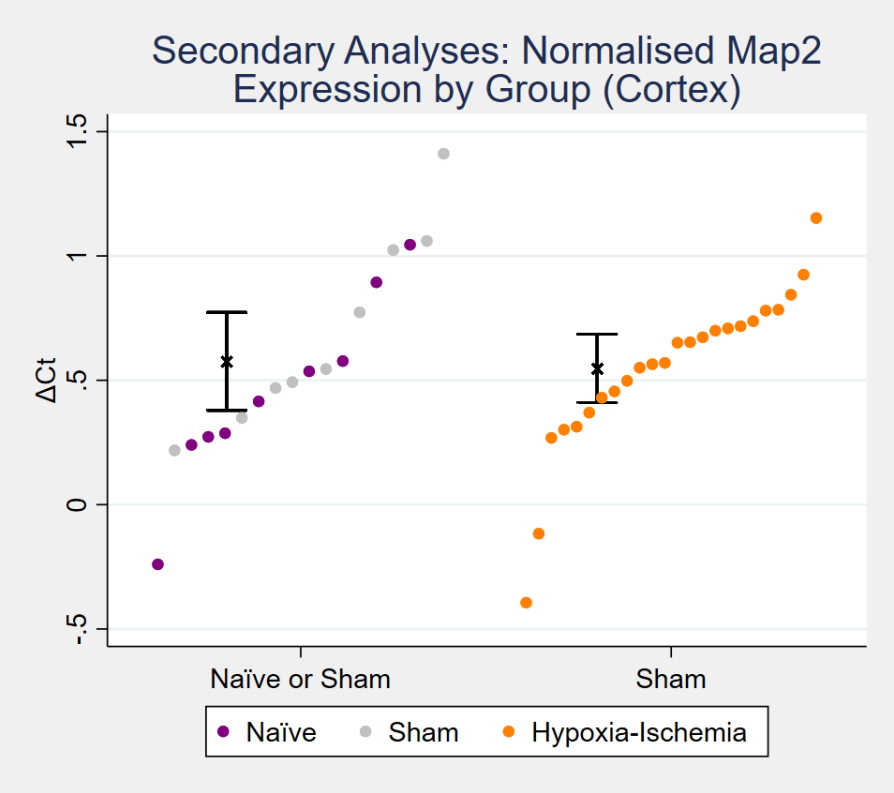


Figure 3.12. *Map2* expression (cortex): plot of secondary analyses



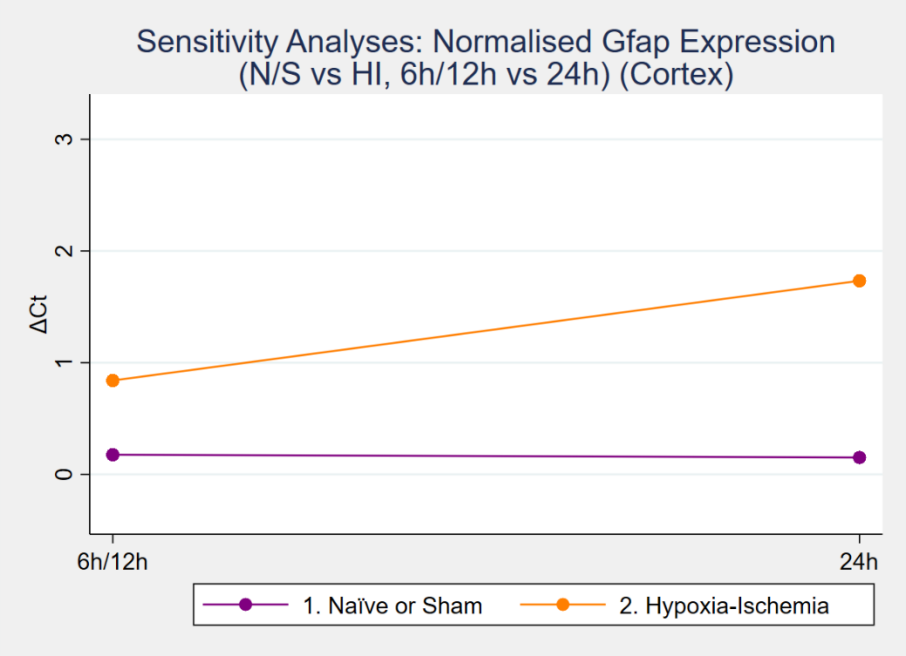
Given the non-significant trend for late *Gfap* upregulation in 24h HI, a sensitivity analysis was carried out merging the 6h and 12h time points in an early (6h + 12h) vs late (24h) design comparing the HI group vs the control group (N/S) (Table 3.7). Evidence of interaction between group and time strengthened (p=0.060), with an effect size of 6% (Figure 3.13. *Gfap* expression (cortex): plot of sensitivity analyses).

Table 3.7. *Gfap* expression (cortex): sensitivity analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	P	Effect size
	Group (N/S vs HI)		1	11.54	22.61	**<0.00001**	**0.35**
Gfap	*Time (6h/12h vs 24h)*	<0.00001	1	1.72	3.37	0.074	0.06
	*Group*Time*		1	1.93	3.77	0.060	0.06

Figure 3.13. *Gfap* expression (cortex): plot of sensitivity analyses



3.4.2.2 Inflammation

Descriptive statistics are reported in Table 3.8. Results from the primary analyses are reported in Table 3.9. Briefly, there was some evidence that all three pro-inflammatory cytokines (*Tnfα*, *Il1β* and *Il6*) were upregulated in the left cortex following HI, with a non-significant trend for early upregulation (i.e. higher at 6h vs later time points).

Table 3.8. Inflammation (Cortex): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time	n	*Tnfα*		*Il1β*		*Il6*	
			Mean ΔCt	SD	Mean ΔCt	SD	Mean ΔCt	SD
	6	3	-8.03	0.25	-10.47	0.53	-10.27	0.14
N	**12**	3	-7.89	0.45	-10.49	0.51	-10.32	0.49
	24	3	-8.18	0.57	-10.49	0.27	-10.36	0.25
	6	3	-8.16	0.44	-10.56	0.60	-10.24	0.51
S	**12**	3	-7.77	0.30	-10.29	0.34	-10.32	0.36
	24	3	-7.75	0.28	-10.95	0.13	-10.50	0.35
	6	8	-6.48	1.10	-7.89	2.56	-8.01	1.37
HI	**12**	8	-7.39	1.20	-9.85	1.17	-9.32	1.67
	24	8	-7.17	0.72	-10.37	0.50	-9.17	1.29

Table 3.9. Inflammation (cortex): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	P	Effect size	*Post hoc* Scheffe's p
Tnfα	*Group (N vs S vs HI)*	0.11	2	4.67	6.40	**0.005**	**0.23**	N (p=0.015) and S (p=0.040) ≠ HI
	Time		2	0.07	0.09	0.911	0 (-0.05)	
	*Group*Time*		4	0.68	0.93	0.457	0 (-0.01)	
Il1β	*Group (N vs S vs HI)*	0.10	2	7.07	3.92	**0.030**	**0.14**	N (p=0.178) and S (p=0.124) may be ≠ HI
	Time		2	2.68	1.49	0.241	0.03	
	*Group*Time*		4	2.80	1.56	0.209	0.06	
Il6	*Group (N vs S vs HI)*	0.43	2	11.60	8.34	**0.001**	**0.29**	N (p=0.010) and S (p=0.008) ≠ HI
	Time		2	0.92	0.66	0.523	0 (-0.02)	
	*Group*Time*		4	0.75	0.54	0.708	0 (-0.05)	

Evidence of a group effect was strong for both *Tnfα* (p=0.005) and *Il6* (p=0.001), with large effect sizes of 23% and 29% respectively, i.e. 23% of the variation in *Tnfα* expression and 29% of the variation in *Il6* expression were accounted for by group (Table 3.9). Evidence was weaker but still significant for *Il1β* (p=0.030), with an effect size of 14% (Table 3.9). *Post hoc* analyses suggested that this was driven by the HI group for all cytokines, with no evidence of differences between naïve and sham. Such upregulation of the cytokines in the ipsilateral cortex is reflected by plots of normalised expression and fold change (Figure 3.14**,** Figure 3.15, Figure 3.16). There was no evidence of a time effect for any of the cytokines in the primary analyses.

The plots of normalised expression show a trend for a bimodal distribution for all cytokines in the HI group. The plots of fold change show that *Tnfα* and *Il6* are higher in the HI group at all time points compared to naïve rats at 6h, whereas *Il1β* upregulation in the HI group is mainly limited to the 6h time point. Indeed, despite the lack of significant interaction effects with current sample size and the high variability in the HI group at 6h (especially for *Il1β* and *Il6*), it can be observed that for all cytokines the largest increase seems to occur at 6h.

Specifically, the HI group at 6h had 3-fold (300%) higher expression of *Tnfα*, 4.8-fold higher expression of *Il6* and 6-fold higher expression of *Il1β* compared to the naïve groups at the corresponding time points. For *Tnfα* and *Il6*, expression in the HI group at 12h and 24h was still 1.4-2.2 fold higher compared to the naïve groups at the corresponding time points.

Figure 3.14. *Tnfα* expression (cortex): plots

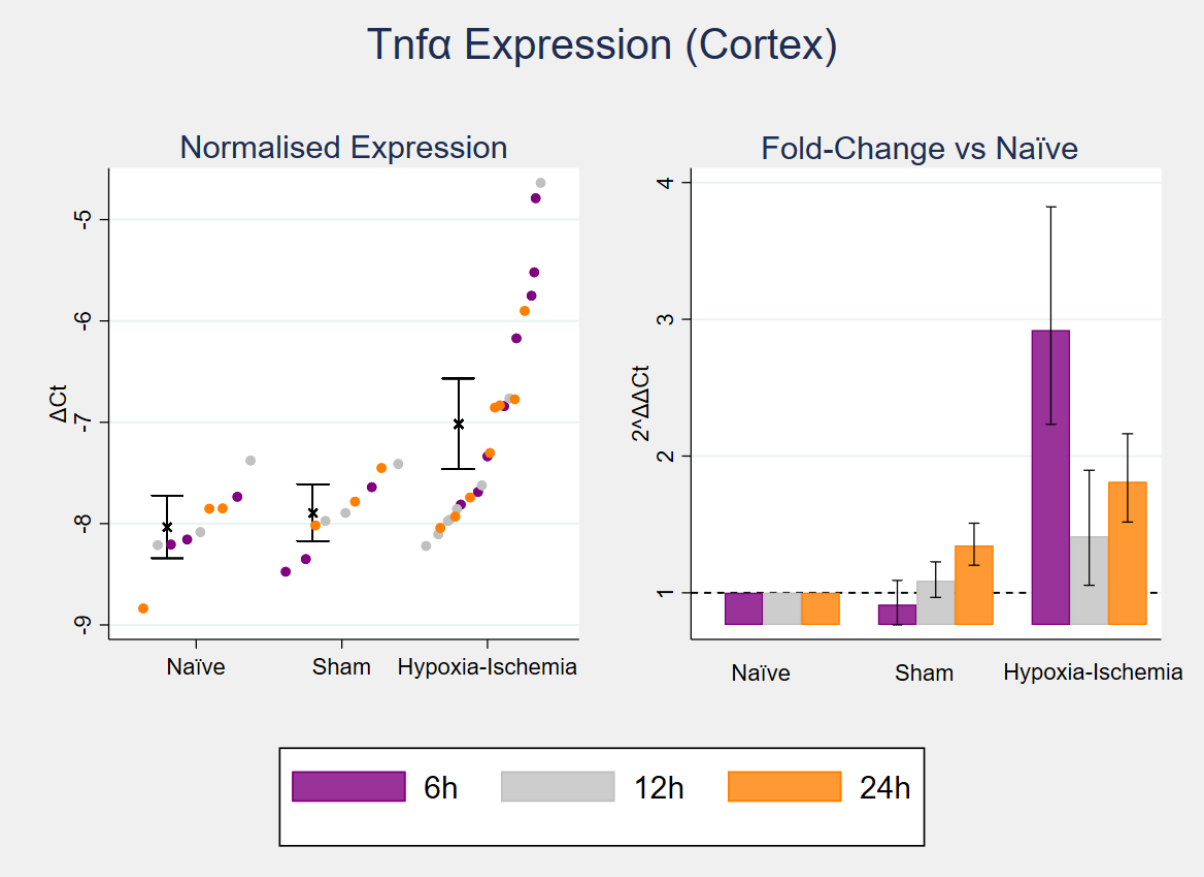


Figure 3.15. *Il6* expression (cortex): plots

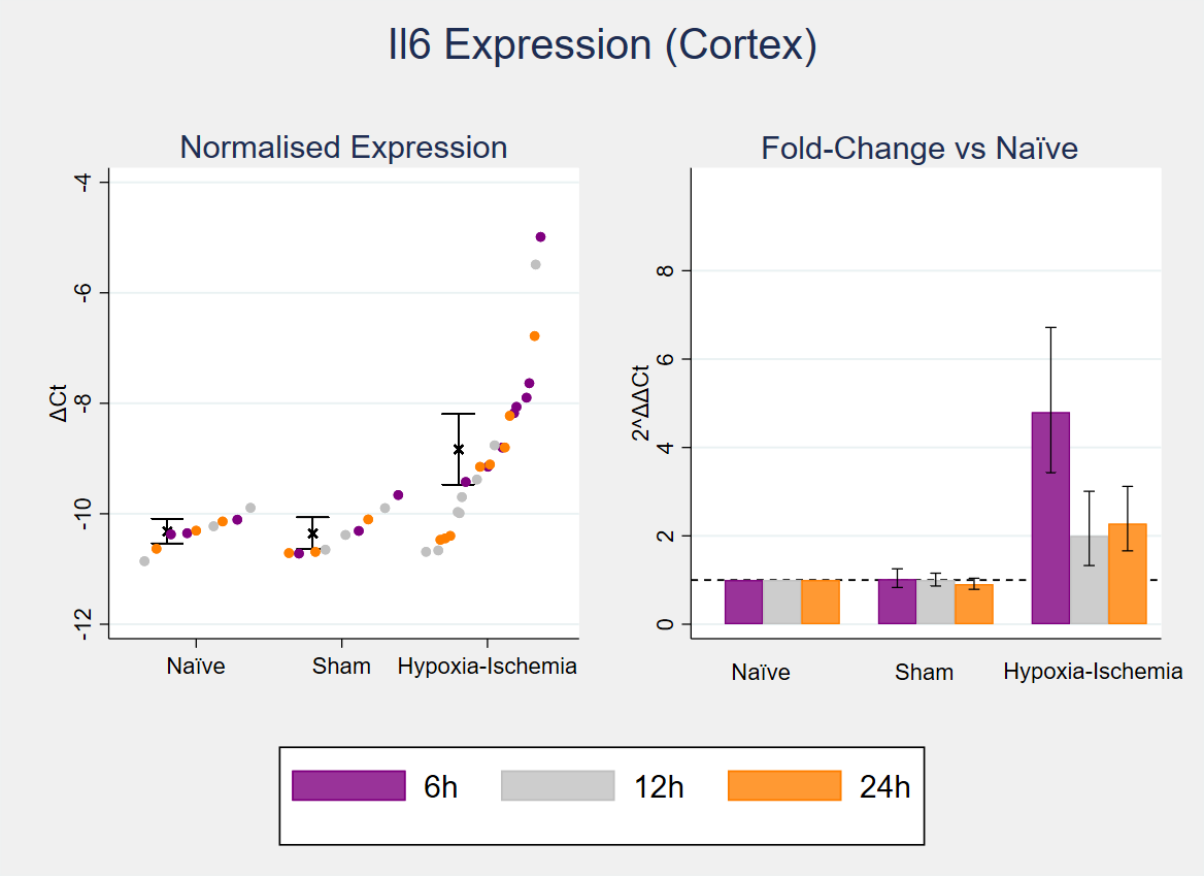
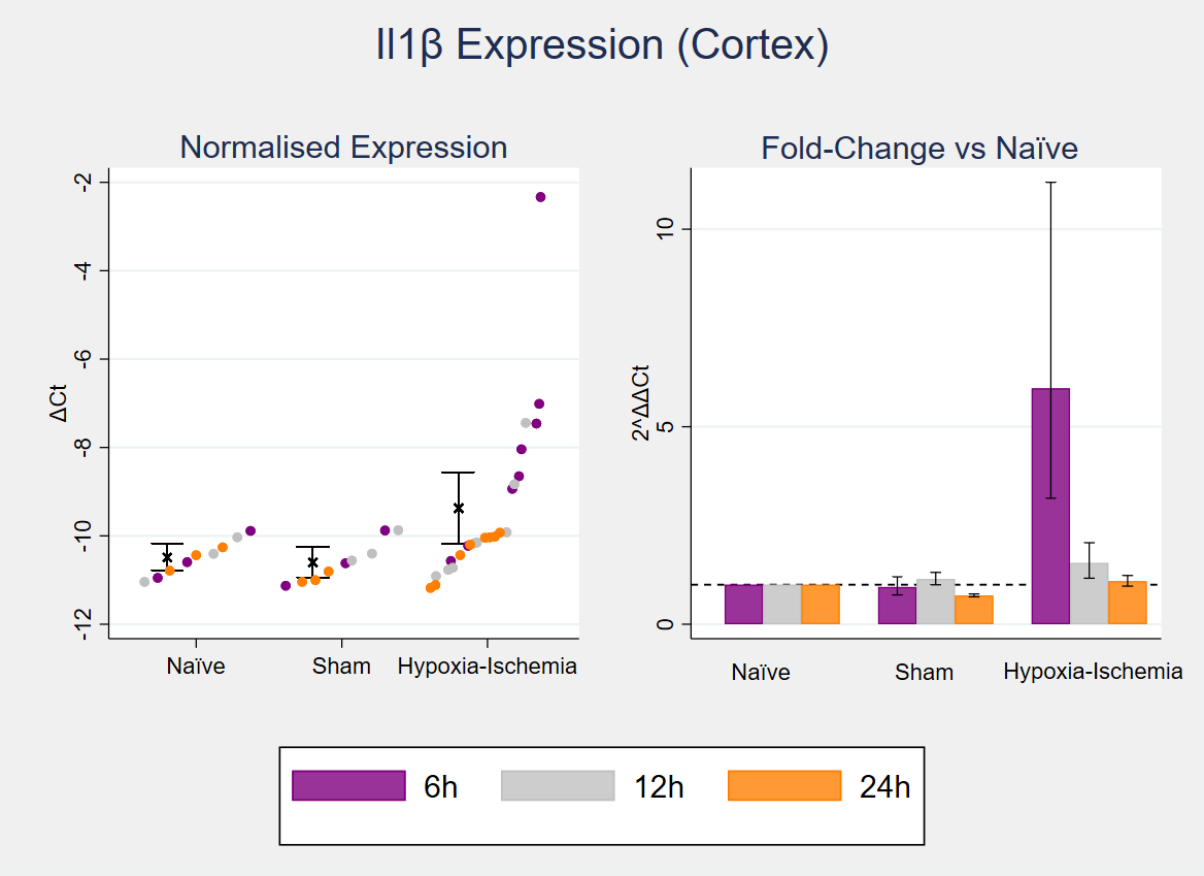


Figure 3.16. Ilβ expression (cortex): plots



In the secondary analyses merging naïve and sham groups, evidence of upregulation in the HI group strengthened for both *Tnfα* (p=0.0007) and *Il6* (p=0.0001), with similar effect sizes of 25% and 31%. Additionally, some evidence an interaction effect emerged for *Il1β* (p=0.049), with an effect size of 10%, driven by the 6h time point (Table 3.10**,** Figure 3.17**,** Figure 3.18**,** Figure 3.19).

Table 3.10. Inflammation (cortex): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Tnfα	*N vs S*	*Group*	0.03	1	0.09	0.56	0.470	0 (-0.03)
		Time		2	0.11	0.68	0.524	0 (-0.03)
		*Group*Time*		2	0.12	0.74	0.496	0 (-0.04)
	N/S vs HI	*Group*		1	9.24	13.66	**0.0007**	**0.25**
		Time		2	0.41	0.61	0.549	0 (-0.02)
		*Group*Time*		2	1.24	0.83	0.174	0 (-0.04)
Il1β	*N vs S*	*Group*	<0.00001	1	0.06	0.32	0.579	0 (-0.05)
		Time		2	0.17	0.92	0.424	0 (-0.01)
		*Group*Time*		2	0.16	0.86	0.446	0 (-0.02)
	N/S vs HI	*Group*	0.79	1	14.07	8.47	0.006	0.16
		Time		2	6.47	3.89	0.030	0.13
		*Group*Time*		2	5.44	3.28	**0.049**	**0.10**
Il6	*N vs S*	*Group*	0.16	1	0.006	0.05	0.834	0 (-0.07)
		Time		2	0.05	0.34	0.721	0 (-0.10)
		*Group*Time*		2	0.01	0.09	0.915	0 (-0.14)
	N/S vs HI	*Group*		1	23.19	18.17	**0.0001**	**0.31**
		Time	0.16	2	2.11	1.65	0.206	0.03
		*Group*Time*		2	1.49	1.17	0.323	0.01

Figure 3.17. *Tnfα* expression (cortex): plot of secondary analyses

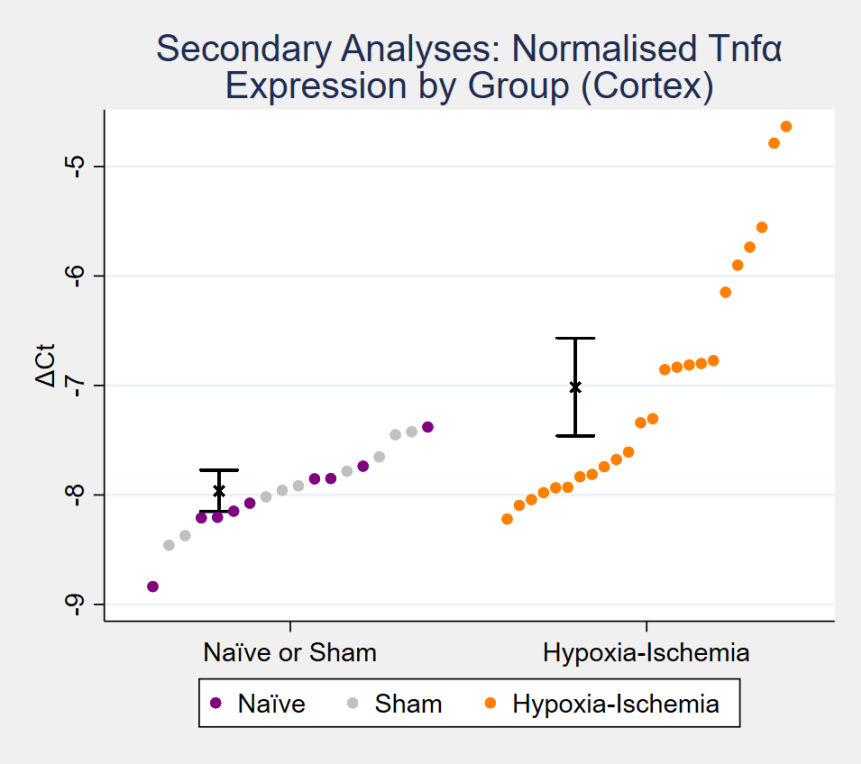


Figure 3.18. Il1β expression (cortex): plot of secondary analyses

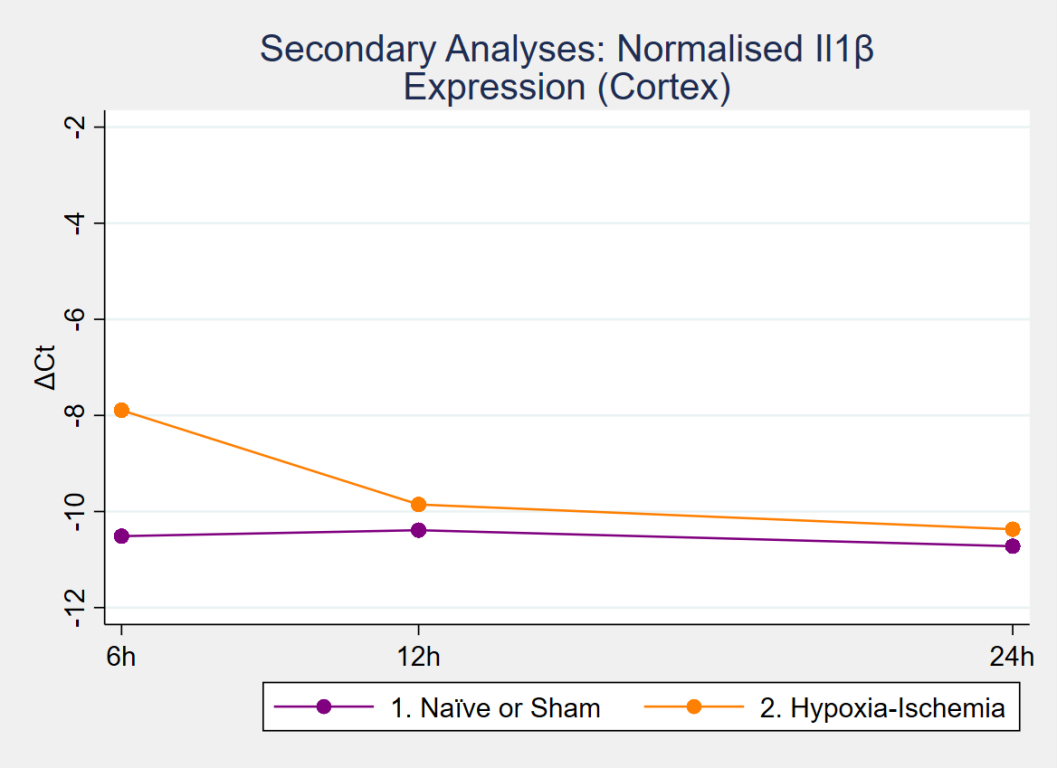
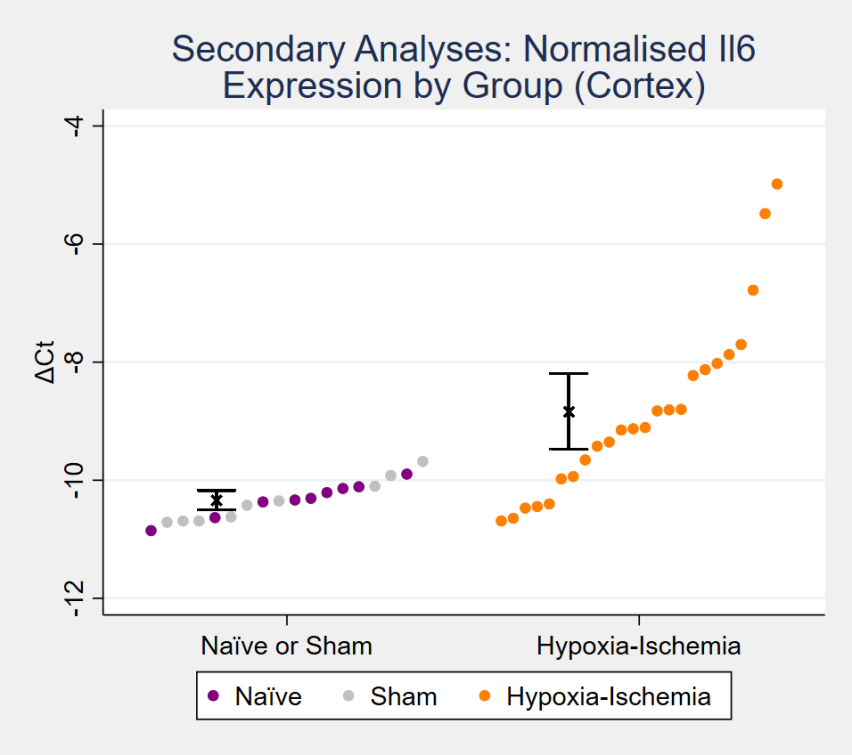


Figure 3.19. *Il6* expression (cortex): plot of secondary analyses



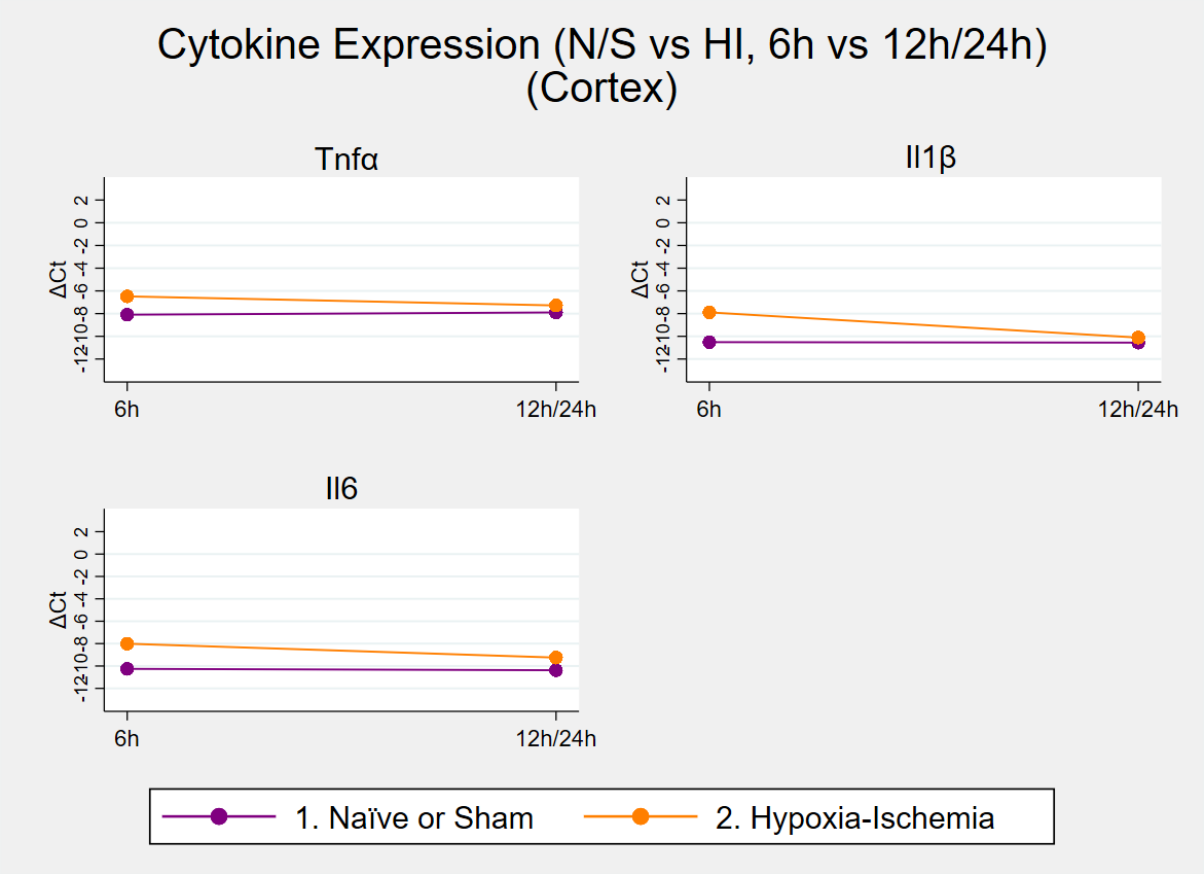
Given the non-significant trend for early upregulation of the cytokines in HI group at 6h, a sensitivity analysis was carried out merging the 12h and 24h time points in an early (6h) vs late (12h + 24h) design comparing the HI group vs control group (N/S) (Table 3.11, Figure 3.20). Evidence for the interaction effect between group and time strengthened for *Il1β* (p=0.013) with an effect size of 13%. There was also a non-significant interaction effect for *Tnfα* (p=0.069), with an effect size of 6%, and weaker evidence for *Il6* (p=0.133) and an effect size of 3%.

Table 3.11. Inflammation expression (cortex): sensitivity analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	P	Effect size
	Group (N/S vs HI)		1	11.34	17.52	**0.0002**	**0.29**
Tnfα	*Time (6h vs 12h/24h)*	0.005	1	0.82	1.26	0.269	0.006
	*Group*Time*		1	2.27	3.50	0.069	0.06
	Group (N/S vs HI)		1	21.47	13.33	0.0008	0.24
Il1β	*Time (6h vs 12h/24h)*	0.01	1	11.70	7.26	0.010	0.14
	*Group*Time*		1	10.83	6.72	**0.013**	**0.13**
	Group (N/S vs HI)		1	26.05	21.49	**<0.00001**	**0.34**
Il6	*Time (6h vs 12h/24h)*	0.03	1	4.21	3.48	0.070	0.06
	*Group*Time*		1	2.86	2.36	0.133	0.03

Figure 3.20. Inflammation expression (cortex): plots of sensitivity analyses



3.4.2.3 Glutamate transporter

Descriptive statistics are reported in Table 3.12. There were no significant changes in *Glt1* expression with group or time. On the other hand, an 8% effect was estimated and *post hoc* analyses produced very weak evidence of differences between the HI group and the sham group (p=0.098) (Table 3.13, Figure 3.21). In the secondary analyses merging naïve and sham, some evidence emerged that *Glt1* may be lower in the HI group (p=0.036) with a small effect size of 9% (Table 3.14, Figure 3.22).

Table 3.12. *Glt1* (cortex): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time	n	*Glt1*	
			Mean ΔCt	SD
N	**6**	3	1.90	0.30
	12	3	2.15	0.39
	24	3	1.95	0.54
S	**6**	3	2.18	0.47
	12	3	2.03	0.60
	24	3	2.25	0.14
HI	**6**	8	1.70	0.58
	12	8	1.89	0.40
	24	8	1.73	0.41

Table 3.13. *Glt1* (cortex): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	p	Effect size	*Post hoc* Scheffe's p
Glt1	*Group (N vs S vs HI)*	0.43	2	0.52	2.50	0.098	0.08	S may be ≠ HI (p=0.094)
	Time		2	0.03	0.13	0.878	0 (-0.05)	
	*Group*Time*		4	0.05	0.26	0.901	0 (-0.08)	

Figure 3.21. *Glt1* expression (cortex): plots

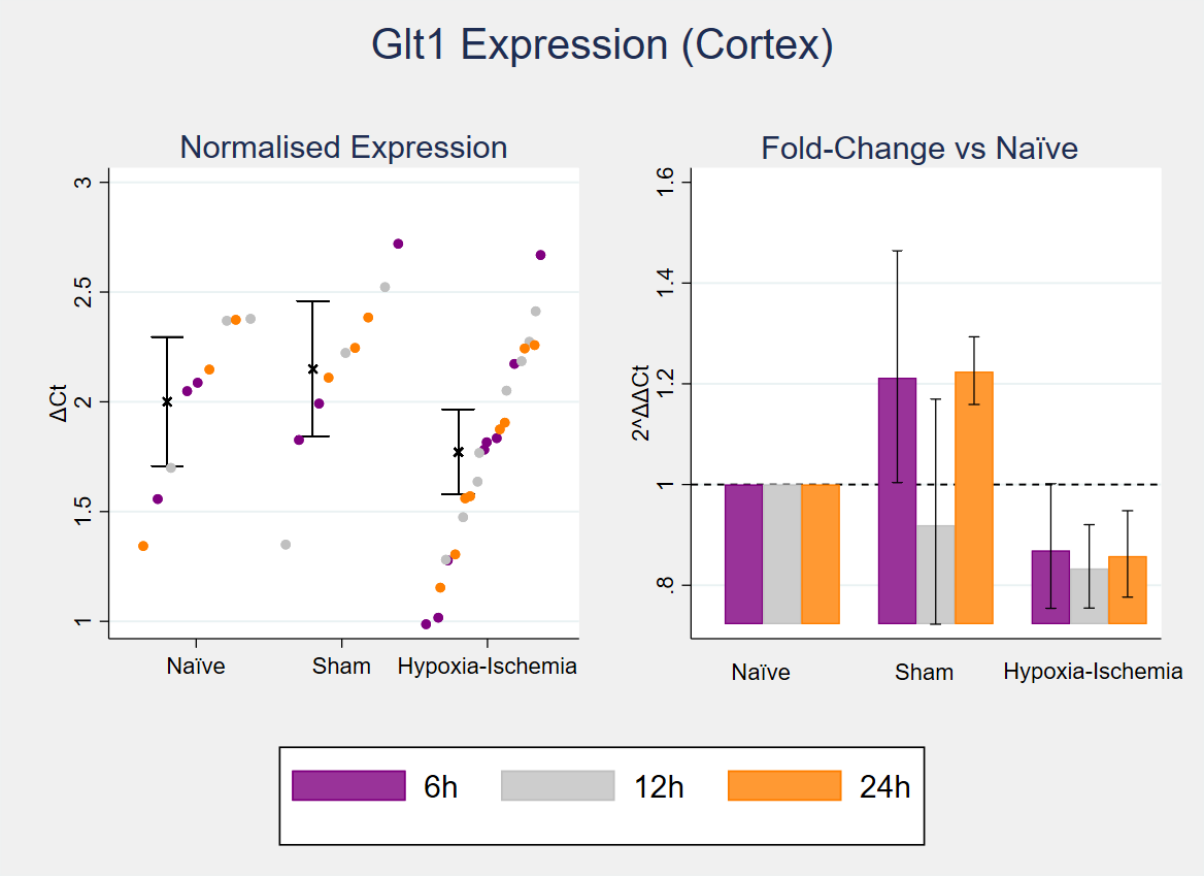
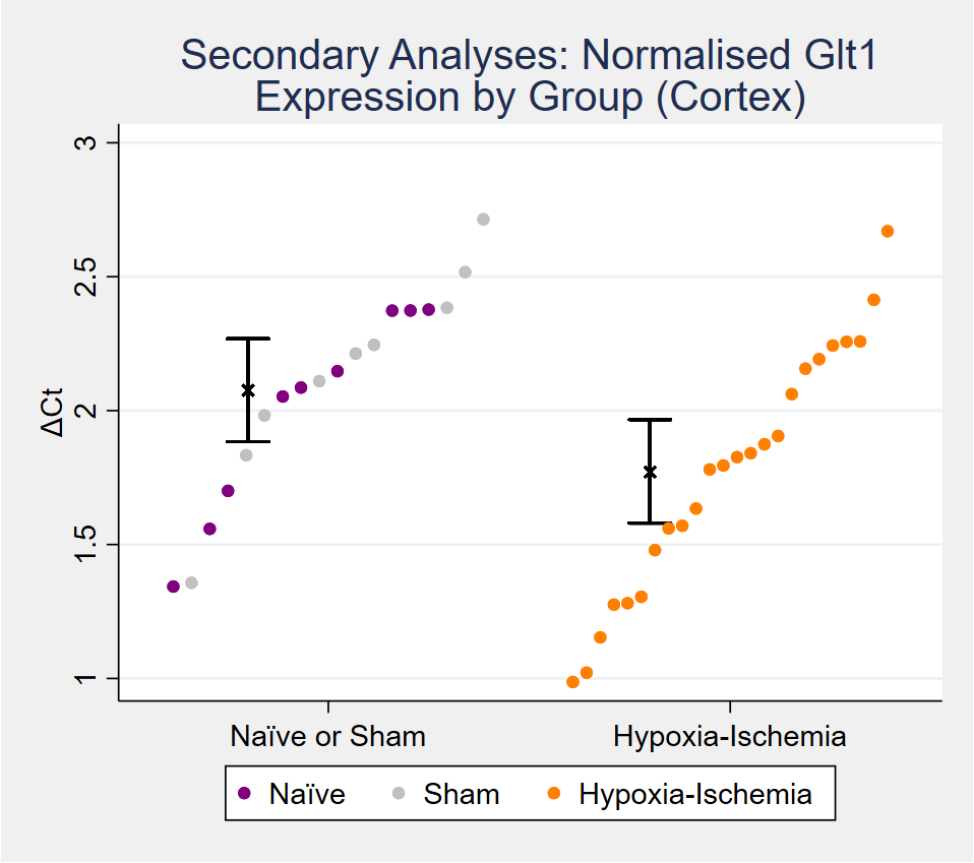


Table 3.14. *Glt1* (cortex): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Glt1	*N vs S*	*Group*	0.18	1	0.10	0.53	0.480	0 (-0.03)
		Time		2	0.01	0.04	0.965	0 (-0.15)
		*Group*Time*		2	0.08	0.44	0.657	0 (-0.08)
	N/S vs HI	*Group*	0.63	1	0.95	4.74	**0.036**	**0.09**
		Time		2	0.05	0.26	0.776	0 (-0.04)
		*Group*Time*		2	0.03	0.13	0.874	0 (-0.05)

Figure 3.22. *Glt1* expression (cortex): plot of secondary analyses

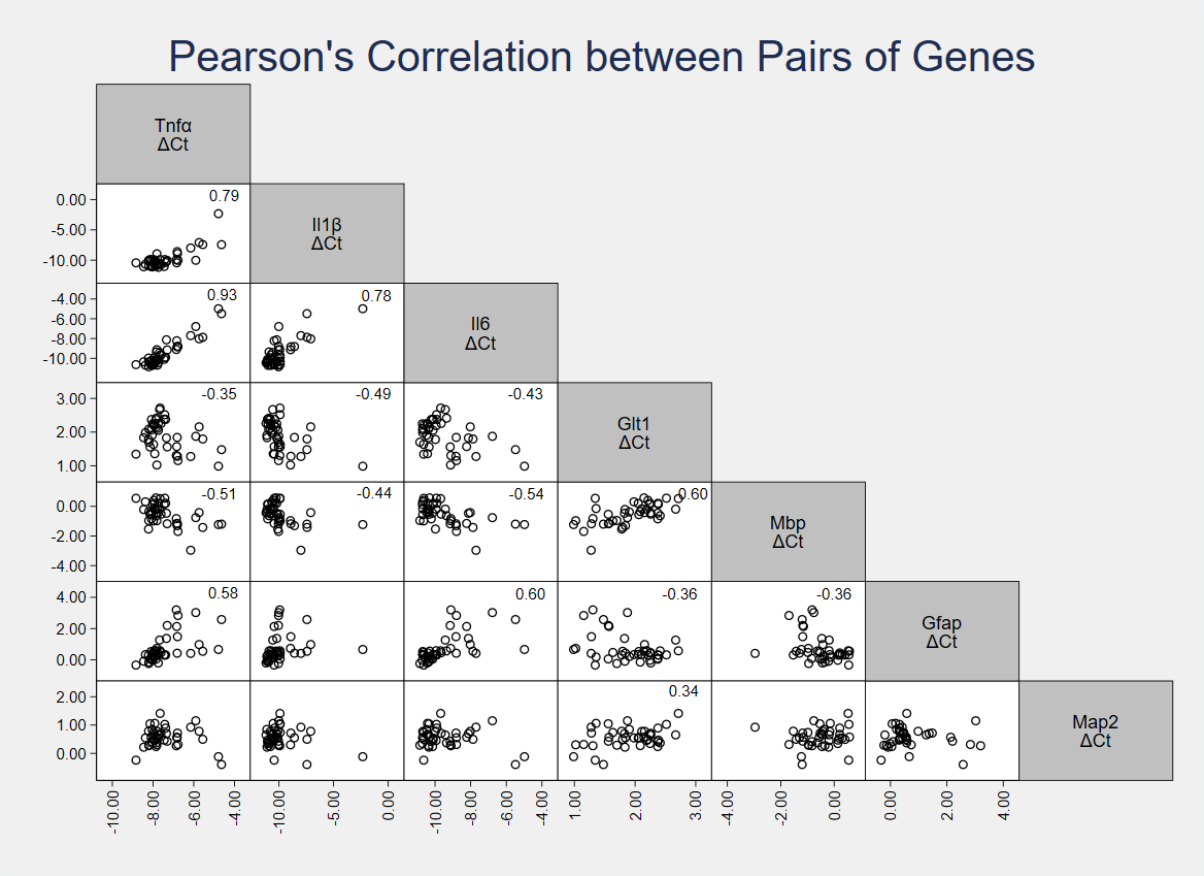


3.4.2.4 Comparison across genes

Correlations between pairs of genes were tested with pairwise Pearson's correlation coefficient and the significant coefficients ($p \leq 0.05$, not corrected for multiple comparisons) annotated on pairwise scatterplots (Figure 3.23). There was a strong positive correlation between all three cytokines (Pearson's correlation coefficients ≥0.8), so that as expression of one cytokine increased so did expression of the others. The correlation was particularly strong between *Tnfα* and *Il6* (Pearson's correlation coefficients =0.9). There was a weak negative correlation between *Glt1* and all cytokines (Pearson's correlation coefficients ≤-0.4). In terms of markers of injury, *Mbp* showed a moderate positive correlation to *Glt1* (Pearson's correlation coefficients =0.6) and a weak/moderate negative correlation to the cytokines (Pearson's correlation coefficients ≤-0.4). *Gfap* showed a moderate positive correlation to *Tnfα* and *Il6* (but not *Il1β*) (Pearson's correlation coefficients = 0.6) and a weak negative correlation to *Glt1* and *Mbp* (Pearson's correlation coefficients =-0.4). *Map2* had a weak positive correlation with *Glt1* only (Pearson's correlation coefficients =0.3).

Figure 3.23. Matrix of pairwise correlations in gene expression (ΔCt) (cortex)

Only correlation coefficients with p≤0.05 are reported.



In sensitivity analyses, the ten pups showing the most extreme normalised expression values (untransformed ΔCt) for each gene were reported in Table 3.15. "Extreme" referred to the highest expression values for *Gfap* and the cytokines, and lowest for *Mbp*, *Map2* and *Glt1*, based on current findings and the direction of change expected from previous literature. Outliers, as seen in the plots, are indicated with a star. The rats with the most extreme expression for one gene (ranked 1st-2nd) tended to be amongst those with most extreme expression for at least 3 other genes (ranked 1st-10th). For example, the pup in the 6h HI group with highest expression of *Il1β* (outlier in Figure 3.16) is also the pup with highest expression of *Il6* (outlier in Figure 3.15) and lowest expression of *Glt1* and amongst the pups with highest *Tnfα* and lowest *Mbp* (in purple). Similarly, the pup with the highest *Tnfα* expression, from the 12h HI group, is amongst the pups with highest expression for *Il6* (outlier in Figure 3.15), *Il1β* and *Gfap* and lowest expression for *Mbp*, *Map2* and *Glt1*. The pup with the lowest expression for *Mbp* (outlier in Figure 3.7), from the 6h HI group, was also amongst the pups with highest cytokine levels and lowest *Glt1* levels (in orange). Since

there was no indication that the outliers were technical artifacts, they were retained in the analyses.

With regard to *Glt1*, the pup with lowest expression (in purple) was from the 6h HI group and also amongst the pups with the highest cytokine levels and lowest *Mbp* and *Map2*. The second in rank for *Glt1* (in yellow), also from the 6h HI group, also had high *Il1β* and low *Map2*. The third in rank (red) was from the HI 24h group and also had high *Tnfα*, *Il6* and *Gfap*, and low *Mbp* and *Map2*. The fourth in rank, from the 6h HI group, also had high levels of all three cytokine and the lowest level of *Mbp*. Therefore, the four pups with lowest *Glt1* expression in the study all belonged to the HI groups and also had particularly extreme levels of at least one cytokine and injury marker compared to the other pups in the study. While some interesting patterns emerge from these data, these are sensitivity analyses from a small pilot study and must be interpreted with caution.

Table 3.15. Top 10 rats ranked by most extreme gene expression for each gene (cortex)

"Extreme": the highest or lowest expression value within the sample, depending on the expected direction of change based on these findings and previous literature, i.e. highest to lowest expression for the cytokines and *Gfap* (▼); lowest to highest expression (▲) for *Glt1*, *Mbp* and *Map2*.

Cell numbers are rat IDs (e.g. 6H5 is the 5th of one of the 8 rats from the hypoxia-ischaemia group at 6h; N: naïve; S: sham; H: hypoxia-ischaemia). Each rat ID has been assigned one colour in the table (e.g. 6H5 is shown in orange). Outliers (see corresponding figures in the main text) are marked with *.

Mbp▲	*Gfap*▼	*Map2*▲	*Tnfα*▼	*Il1β*▼	*Il6*▼	*Glt1*▲
6H5*	24H2	12H4	12H4	6H2*	6H2*	6H2
24H8	24H4	24N3	6H2	6H8	12H4*	6H1
12H8	24H8	6H2	6H6	6H6	24H4	24H8
6H6	12H4	6S2	6H8	12H4	6H5	6H5
6H4	24H3	24N2	24H4	6H5	6H6	12H1
6H2	24H7	24H2	6H5	6H4	6H8	24H2
12H4	12H1	6N2	24H8	12H1	6H3	24N3
12H1	6H3	12N1	12H1	6H1	24H7	12S1
24H3	6H7	6H1	6H4	12H3	24H8	12H4
24H7	6H8	24H8	24H7	6N1	6H4	6N1

3.4.3 Hippocampus

3.4.3.1 Markers of injury

Descriptive statistics are reported in Table 3.16. Results from the primary analyses are reported in Table 3.17. Briefly, there was strong evidence of upregulation of *Gfap*, a marker of astrogliosis, in the HI group (independently of time). There was also strong evidence that *Mbp* was lower at 6h compared to 24h; this time effect was independent of group, although secondary analyses suggested that *Mbp* may be lost in the HI group compared to the naïve/sham control group. There was no evidence of changes in neuronal marker *Map2* with group or time.

Table 3.16. Markers of Injury (Hippocampus): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time	n	*Mbp*		*Gfap*		*Map2*	
			Mean ΔCt	SD	Mean ΔCt	SD	Mean ΔCt	SD
	6	3	-0.54	1.05	0.80	0.30	0.14	0.32
N	**12**	3	0.52	0.86	0.90	0.54	-0.01	0.23
	24	3	0.56	1.68	0.48	0.58	0.04	0.43
	6	3	-0.83	0.78	0.74	0.20	-0.07	0.12
S	**12**	3	-0.42	0.58	0.37	0.40	-0.11	0.39
	24	3	1.05	0.25	0.69	0.29	-0.19	0.25
	6	8	-0.93	0.89	1.45	0.37	0.01	0.28
HI	**12**	8	-0.66	0.89	1.15	0.66	-0.16	0.17
	24	8	0.03	0.48	1.33	0.82	-0.26	0.14

Table 3.17. Markers of injury (hippocampus): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	p	Effect size	*Post hoc* Scheffe's p
	Group (N vs S vs HI)		2	1.86	2.56	0.093	0.08	N (p=0.187) may be ≠ HI
Mbp	*Time*	0.07	2	4.94	6.78	**0.003**	**0.24**	6h ≠ 24h (p=0.004); 12h may be ≠ 24h (p=0.110)
	*Group*Time*		4	0.53	0.73	0.577	0 (-0.03)	
Gfap	*Group (N vs S vs HI)*	0.24	2	2.17	6.70	**0.004**	**0.24**	N (p=0.033) and S (p=0.008) ≠ HI
	Time		2	0.12	0.38	0.689	0 (-0.04)	

	*Group*Time*		4	0.13	0.41	0.803	0 (-0.07)
Map2	*Group (N vs S vs HI)*	0.07	2	0.13	2.10	0.138	0.06
	Time		2	0.08	1.34	0.277	0.02
	*Group*Time*		4	0.02	0.27	0.895	0 (-0.08)

The naïve group showed particularly high variability for *Mbp*, indeed the highest variability amongst all genes in both brain regions (Figure 3.24). There was strong evidence of a time effect on *Mbp* expression (p=0.003), with a large effect size of 24%, i.e. 24% of the variation in *Mbp* expression was accounted for by time (Table 3.17). *Post hoc* analyses confirmed that expression was significantly lower at 6h compared to 24h independently of group, with weaker evidence for an intermediate effect of 12h. The evidence of a group effect was very weak (p=0.093), with an effect size of 8%. *Post hoc* analyses suggested that *Mbp* may be lost in the HI group compared to naïve rats. In the plot of normalised expression by group, a trend can be seen for lower *Mbp* expression in the sham and HI 6h groups compared to the corresponding 24h groups; this was more ambiguous for the naïve group (Figure 3.24). The time effect is more easily observed in a plot of normalised expression by time (Figure 3.25).

Evidence of a time effect for *Mbp* persisted in the secondary analyses (N vs S: p=0.062; N/S vs HI: p=0.002), maintaining a similar effect size to the primary analyses (24-25%). Moreover, stronger evidence that *Mbp* may be lower in the HI group emerged when merging naïve and sham rats (p=0.035), also maintaining similar effect size (9%) (Table 3.18, Figure 3.28).

Figure 3.24. *Mbp* expression (hippocampus): plots

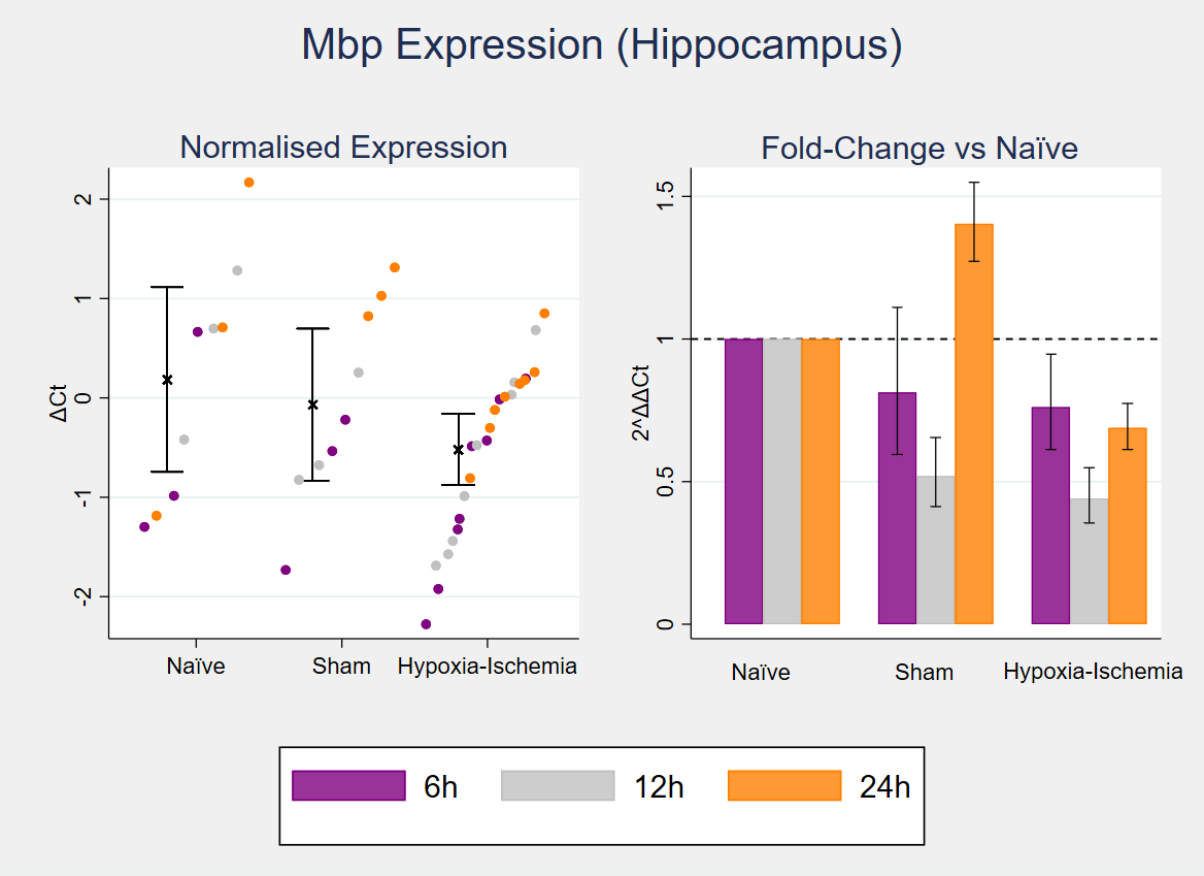
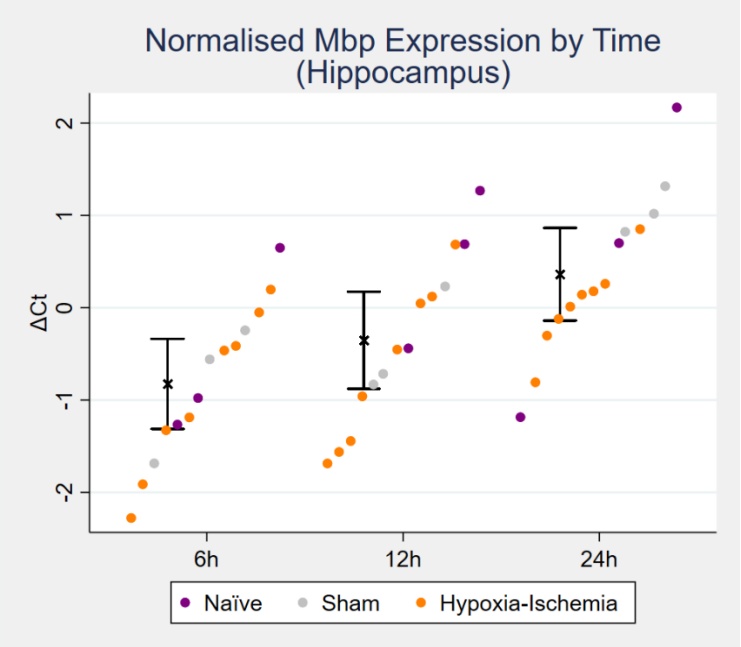
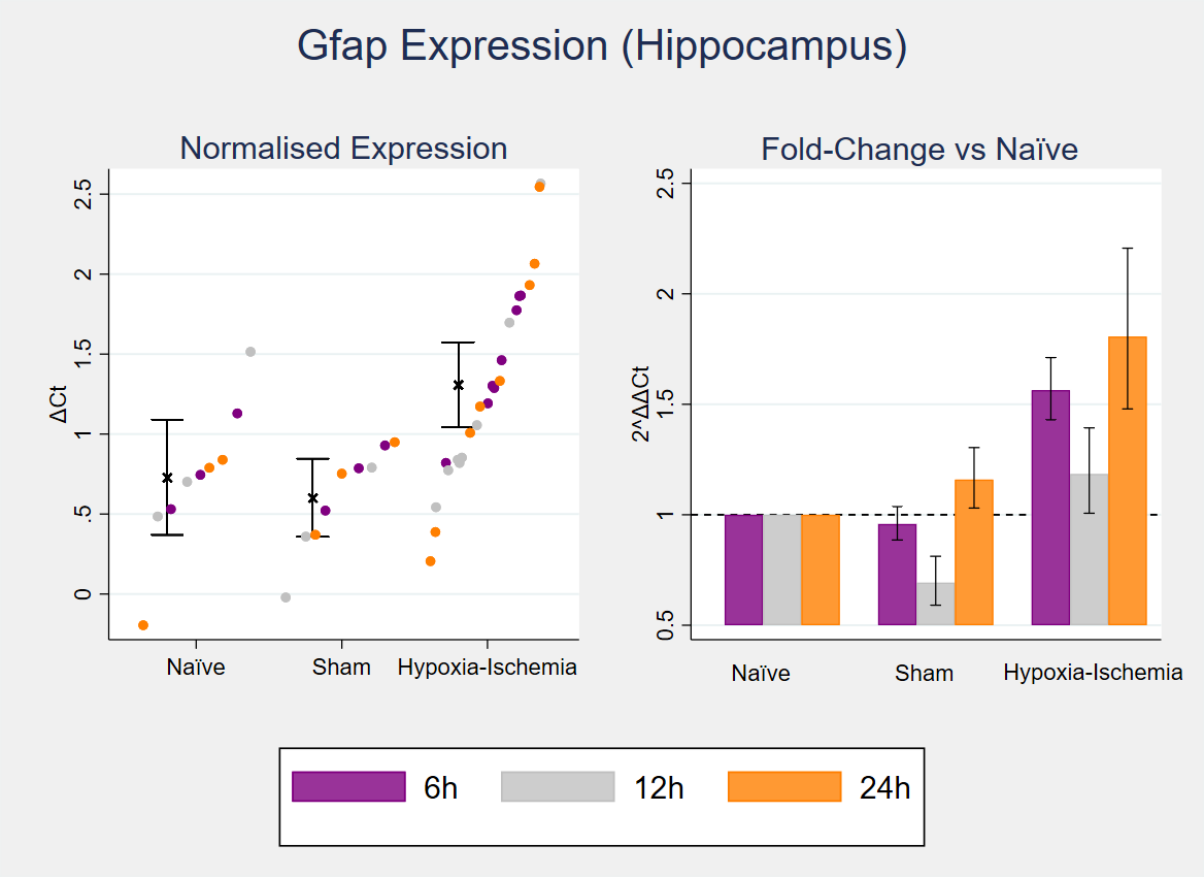


Figure 3.25. *Mbp* expression (hippocampus): plot by time



There was also strong evidence of a group effect on *Gfap* expression (p=0.004) independent of time, with a large effect size of 24%. This was driven by the HI group being different from both naïve and sham groups, as shown by *post hoc* analyses (Table 3.17). The upregulation of *Gfap* in the HI group is reflected in the plots of normalised expression and fold change. The latter shows that *Gfap* expression is 1.2-1.8-fold higher in the HI group at all time points compared to the naïve groups at the corresponding time points (Figure 3.26). A bimodal distribution in the HI group was less obvious in the hippocampus than in the cortex. Unlike in the cortex, in the hippocampus there was no obvious trend for higher expression at 24h in the HI group compared to 6h and 12h. In the secondary analyses merging naïve and sham groups, evidence of *Gfap* upregulation in the HI group strengthened (p=0.0007) and maintained a similar effect size (25%) (Table 3.18).

Figure 3.26. *Gfap* expression (hippocampus): plots



There were no differences in neuronal marker *Map2* in the primary or secondary analyses, and effect sizes were also negligible (Table 3.17**,** Figure 3.27**,** Table 3.18**,** Figure 3.30).

Figure 3.27. *Map2* expression (hippocampus): plots

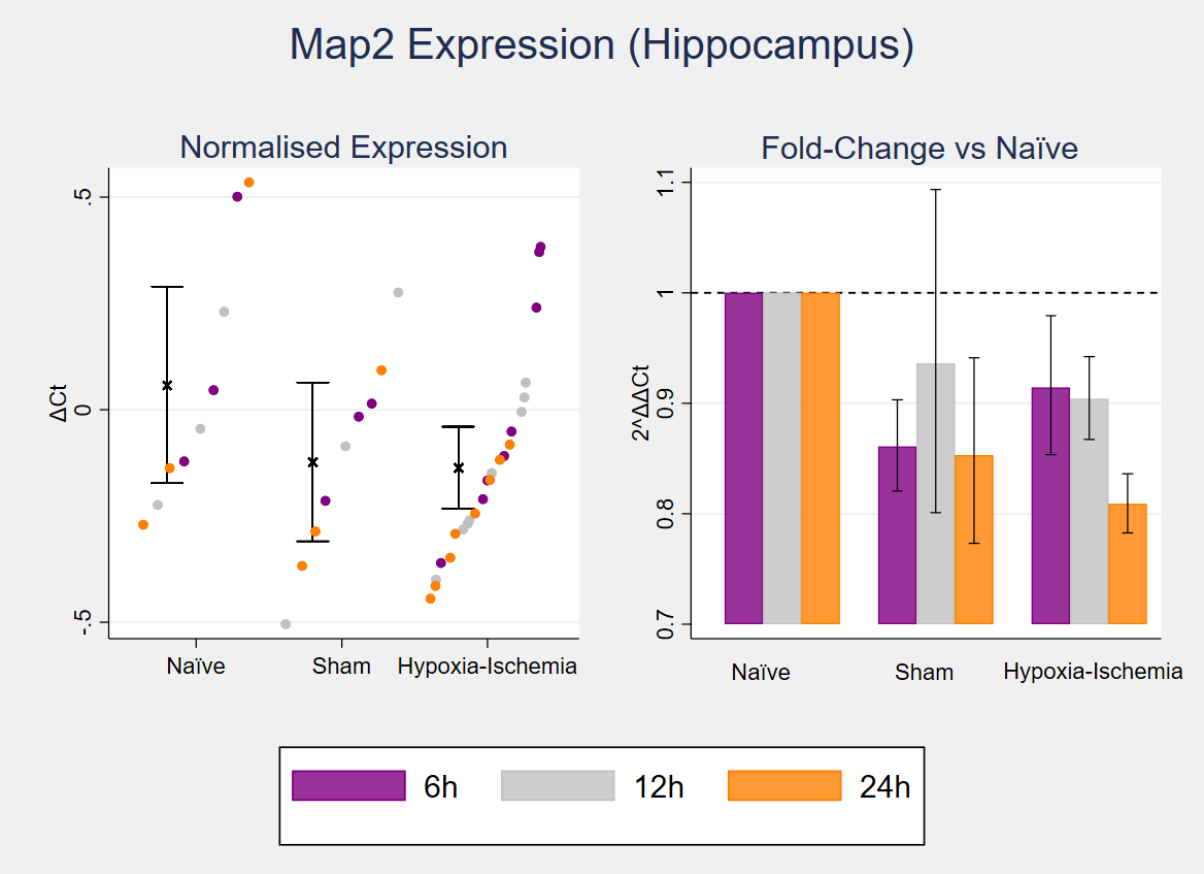


Table 3.18. Markers of injury (hippocampus): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Mbp	*N vs S*	*Group*	0.22	1	0.28	0.30	0.594	0 (-0.05)
		Time		2	3.35	3.54	**0.062**	**0.25**
		*Group*Time*		2	0.77	0.82	0.465	0 (-0.02)
	N/S vs HI	*Group*	0.47	1	3.44	4.79	**0.035**	**0.09**
		Time		2	5.20	7.24	**0.002**	**0.24**
		*Group*Time*		2	0.29	0.41	0.669	0 (-0.03)
Gfap	*N vs S*	*Group*	0.26	1	0.07	0.43	0.526	0 (-0.04)
		Time		2	0.06	0.34	0.720	0 (-0.10)
		*Group*Time*		2	0.21	1.26	0.318	0.03
	N/S vs HI	*Group*	0.13	1	4.27	13.73	**0.0007**	**0.25**
		Time		2	0.17	0.56	0.577	0 (-0.02)
		*Group*Time*		2	0.05	0.16	0.849	0 (-0.04)
Map2	*N vs S*	*Group*	0.40	1	0.15	1.55	0.237	0.04
		Time		2	0.02	0.22	0.808	0 (-0.12)
		*Group*Time*		2	0.01	0.09	0.918	0 (-0.14)
	N/S vs HI	*Group*	0.11	1	0.11	1.82	0.186	0.02
		Time		2	0.13	2.19	0.127	0.06
		*Group*Time*		2	0.02	0.41	0.666	0 (-0.03)

Figure 3.28. *Mbp* expression (hippocampus): plot of secondary analyses

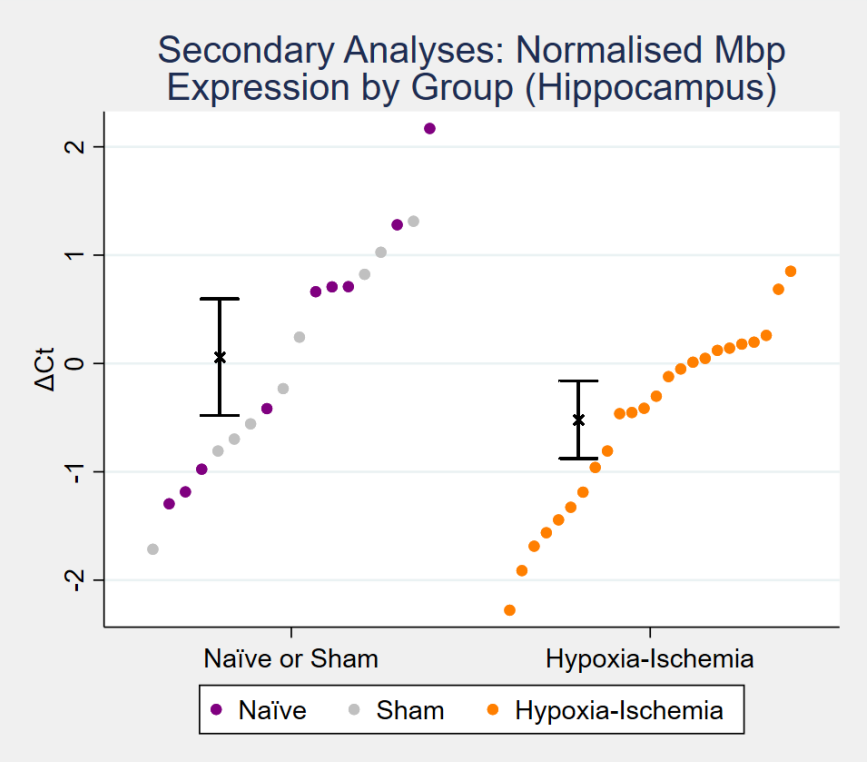


Figure 3.29. *Gfap* expression (hippocampus): plot of secondary analyses

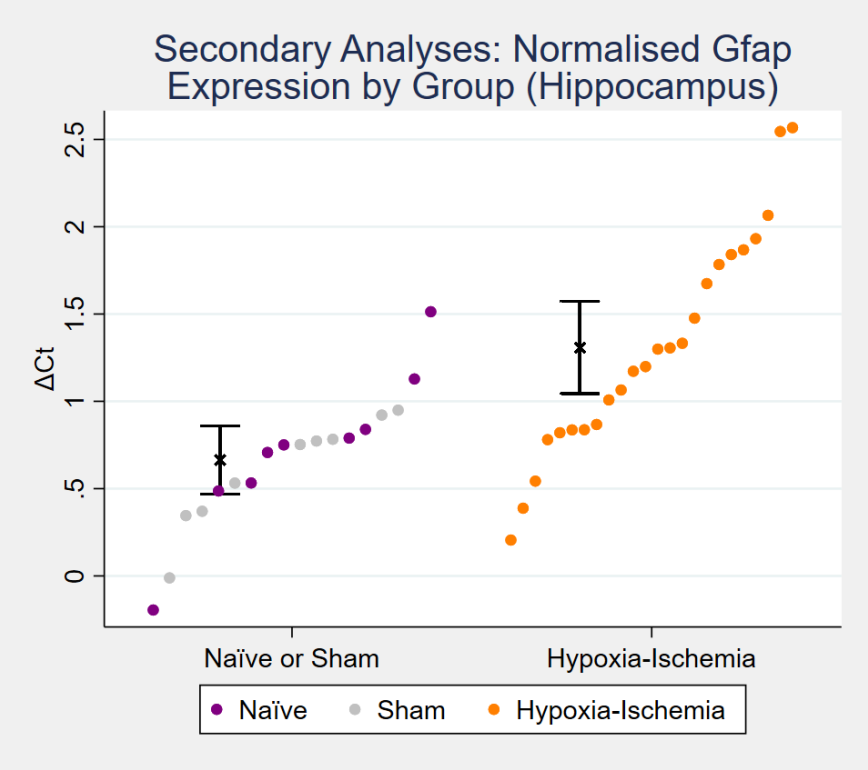
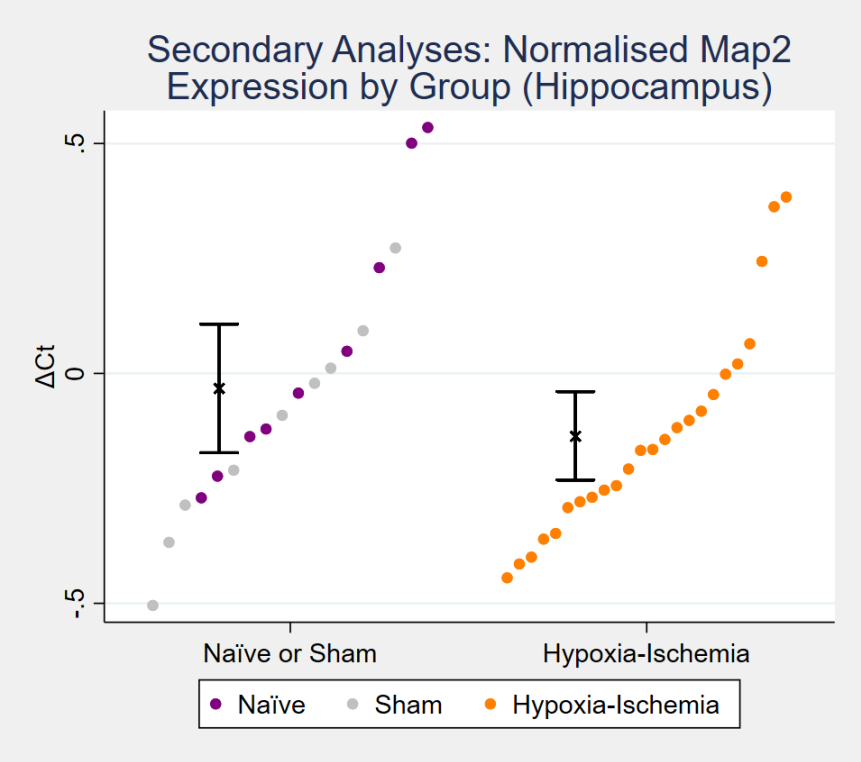


Figure 3.30. *Map2* expression (hippocampus): plot of secondary analyses



3.4.3.2 Inflammation

Descriptive statistics are reported in Table 3.19. Results from the primary analyses are reported in Table 3.20. Briefly, there was some evidence that all three pro-inflammatory cytokines *Tnfα*, *Il1β* and *Il6* were upregulated in the left hippocampus following HI, with a non-significant trend for early upregulation (i.e. higher at 6h vs 12h and 24h).

Evidence of a group effect was strong for *Il6* (p=0.001), with a large effect size of 29% (Table 3.20). Evidence was weaker but still significant for both *Tnfα* (p=0.054) and *Il1β* (p=0.027), with effect sizes of 11% and 14% respectively (Table 3.20). *Post hoc* analyses suggested that this was driven by the HI group for all cytokines, with no evidence of differences between naïve and sham. There was no evidence of a time effect for any cytokine in the primary analyses.

Table 3.19. Inflammation (hippocampus): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time	n	*Tnfα*		*Il1β*		*Il6*	
			Mean ΔCt	**SD**	**Mean ΔCt**	**SD**	**Mean ΔCt**	**SD**
N	**6**	3	-8.10	0.18	-10.24	0.33	-11.05	0.35
	12	3	-7.96	0.18	-10.07	0.20	-10.66	0.12
	24	3	-7.78	0.17	-10.49	0.34	-10.57	0.22
S	**6**	3	-7.85	0.17	-10.77	0.41	-11.13	0.34
	12	3	-8.10	0.21	-10.24	0.32	-11.28	0.83
	24	3	-7.97	0.23	-10.58	0.31	-10.95	0.15
HI	**6**	8	-6.66	1.33	-8.08	2.44	-8.86	1.51
	12	8	-7.57	0.96	-9.64	0.92	-10.05	1.21
	24	8	-7.76	0.51	-10.26	0.31	-10.08	0.91

Table 3.20. Inflammation (hippocampus): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	P	Effect size	*Post hoc* Scheffe's p
Tnfα	*Group (N vs S vs HI)*	0.18	2	2.03	3.18	**0.054**	**0.11**	N (p=0.179) and S (p=0.156) may be ≠ HI
	Time		2	0.40	0.63	0.540	0 (-0.02)	
	*Group*Time*		4	0.70	1.09	0.376	0.01	
Il1β	*Group (N vs S vs HI)*	0.02	2	6.05	4.03	**0.027**	**0.14**	N (p=0.215) and S (p=0.085) may be ≠ HI
	Time		2	1.60	1.07	0.355	0.004	
	*Group*Time*		4	2.41	1.61	0.196	0.06	
Il6	*Group (N vs S vs HI)*	0.16	2	8.66	8.40	**0.001**	**0.29**	N (p=0.036) and S (p=0.004) ≠ HI
	Time		2	0.29	0.28	0.757	0 (-0.04)	
	*Group*Time*		4	1.23	1.20	0.331	0.02	

The cytokine upregulation in the HI group is reflected in the plots of normalised expression and the plots of fold change (Figure 3.31**,** Figure 3.32**,** Figure 3.33). The former shows a bimodal distribution for all cytokines in the HI group. The latter show that *Il6* is higher in the HI group at all time points compared to the age-matched controls, whereas *Tnfα* and *Il1β* upregulation is mainly limited to 6h. Indeed, despite the lack of significant interaction effects with current sample size and the high variability in the HI group at 6h (especially for *Il1β* and *Il6*), the largest increase occurs at 6h it for all cytokines. Specifically, the 6h HI group had 2.7-fold (270%) higher expression of *Tnfα*, 4.5-fold higher expression of *Il6* and 4.5-fold higher expression of *Il1β* compared to the 6h naïve group. For *Il6*, expression at 12h and 24h in the HI group was still 1.5-fold higher than the respective age-matched controls.

Figure 3.31. *Tnfα* expression (hippocampus): plots

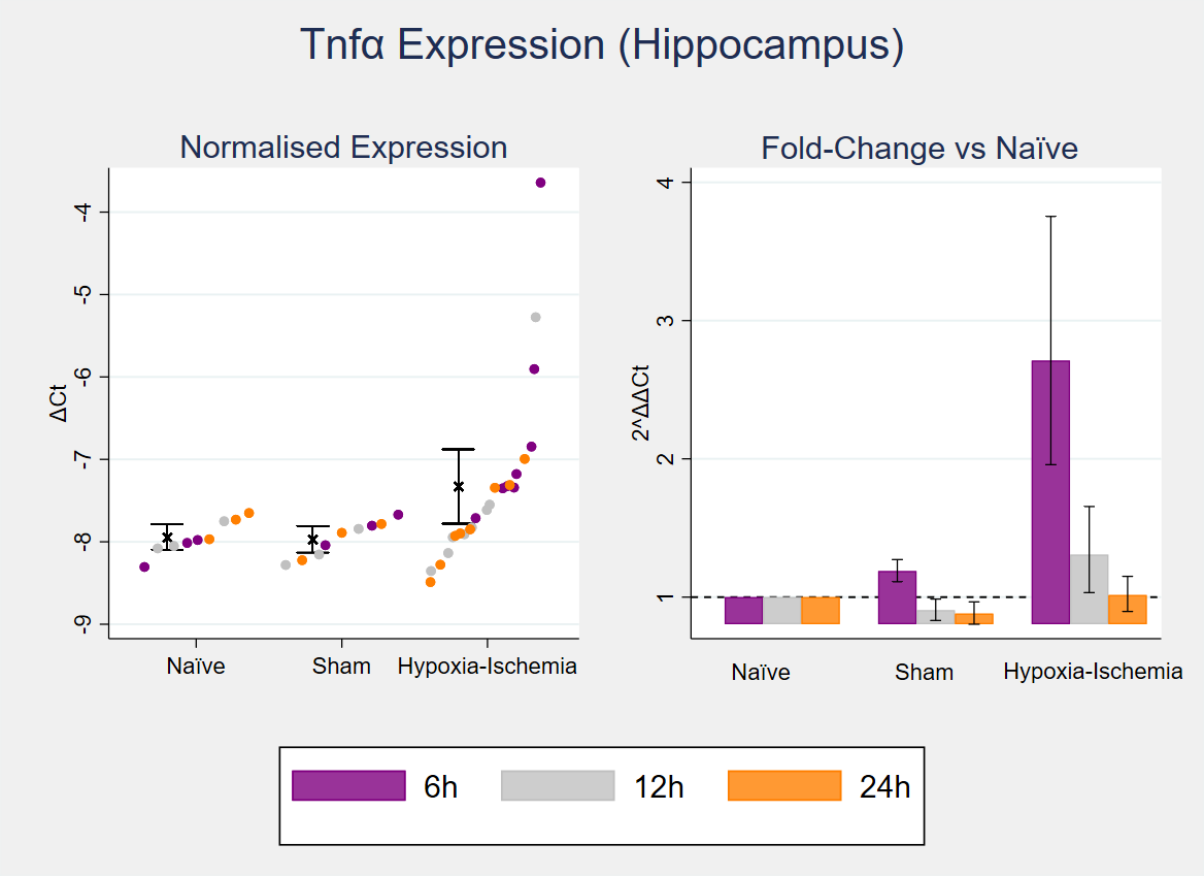


Figure 3.32. Ilβ expression (hippocampus): plots

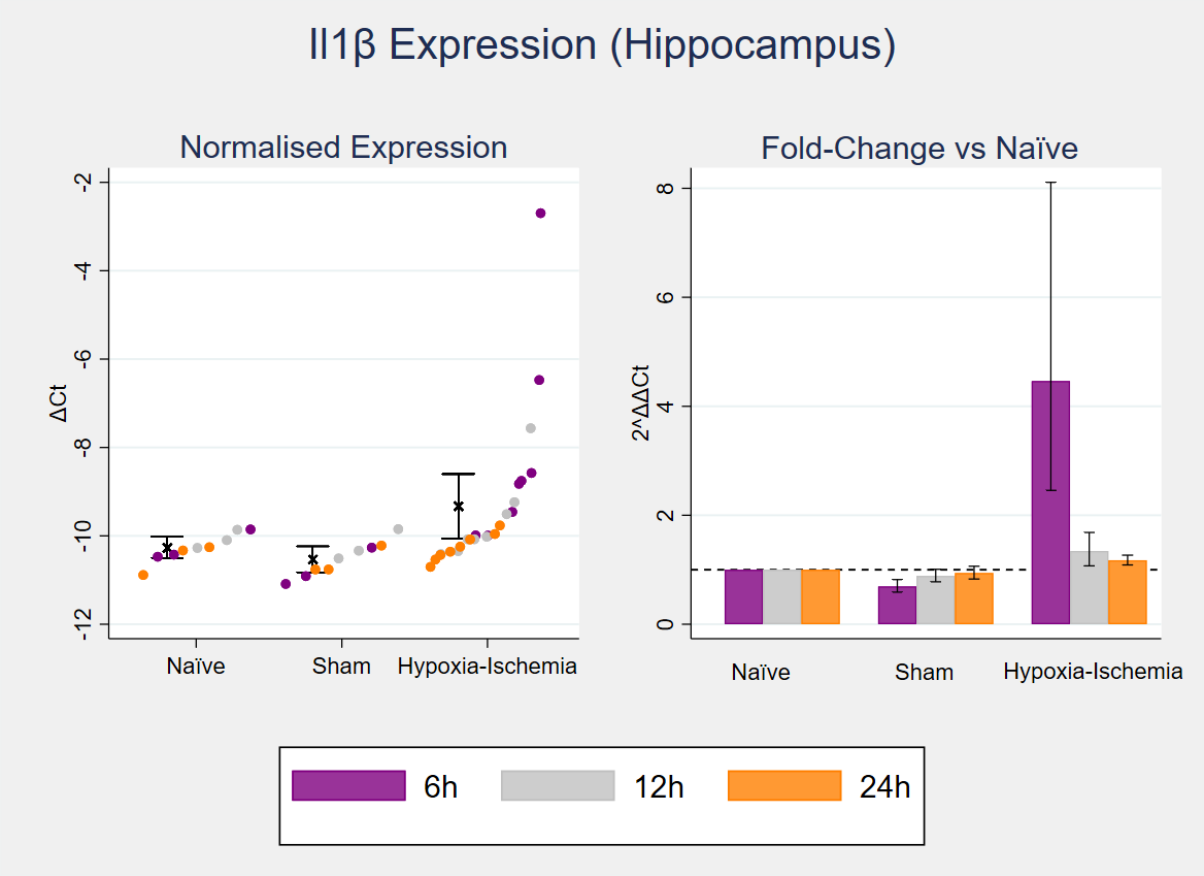
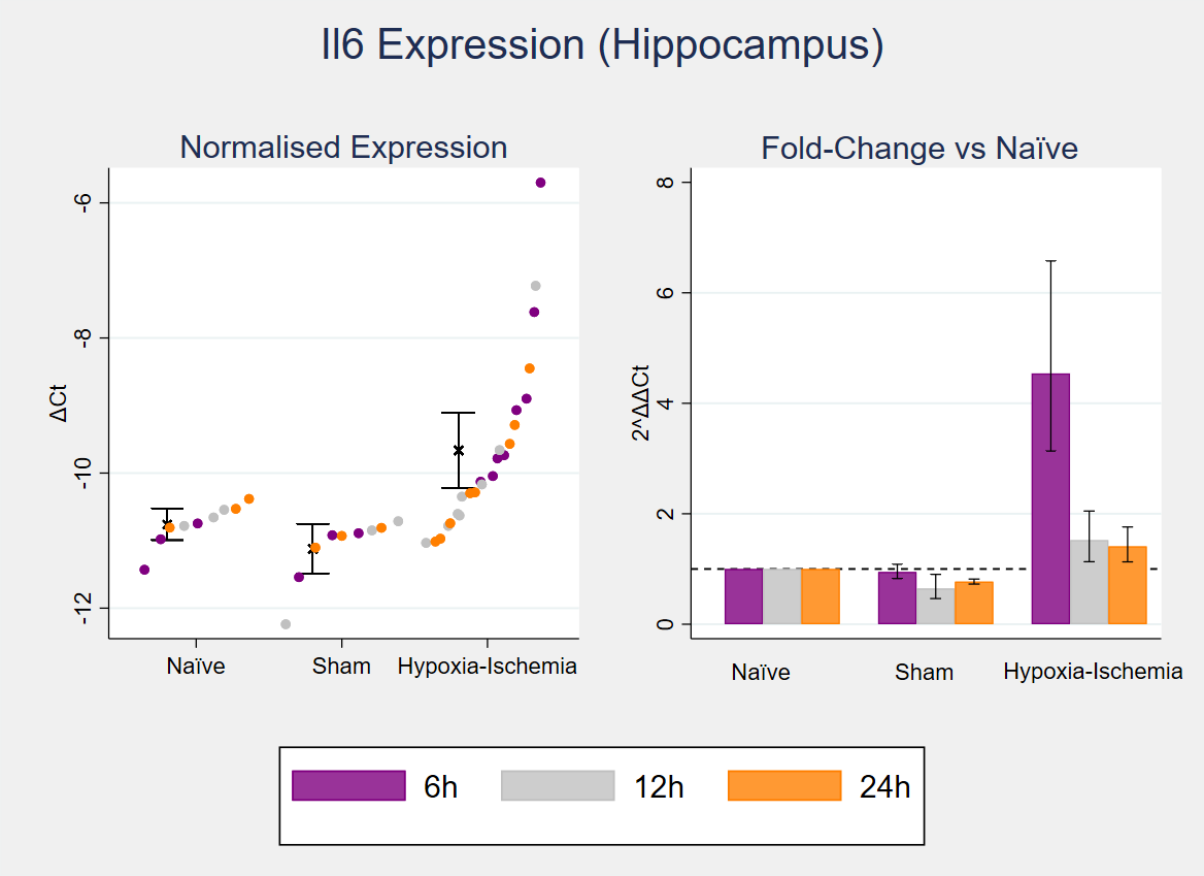


Figure 3.33. *Il6* expression (hippocampus): plots



In the secondary analyses merging naïve and sham groups, evidence of upregulation strengthened for both *Tnfα* (p=0.013) and *Il6* (p=0.0002), with similar effect sizes of 13% and 30% respectively. Additionally, some evidence an interaction effect emerged for *Il1β* (p=0.044), with an effect size of 11%, driven by the 6h time point (Table 3.21**,** Figure 3.34**,** Figure 3.35**,** Figure 3.36).

Table 3.21. Inflammation (hippocampus): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Tnfα	*N vs S*	*Group*	0.94	1	0.003	0.08	0.781	0 (-0.07)
		Time		2	0.04	1.00	0.397	0 (-0.0002)
		*Group*Time*		2	0.09	2.36	0.136	0.15
	N/S vs HI	*Group*	0.04	1	4.06	6.88	**0.013**	**0.13**
		Time		2	1.12	1.90	0.164	0.04
		*Group*Time*		2	1.31	2.22	0.123	0.06
Il1β	*N vs S*	*Group*	0.61	1	0.31	2.91	0.114	0.12
		Time		2	0.27	2.48	0.125	0.17
		*Group*Time*		2	0.08	0.75	0.493	0 (-0.03)
	N/S vs HI	*Group*	0.003	1	11.78	8.49	0.006	0.16
		Time		2	4.18	3.01	0.062	0.09
		*Group*Time*		2	4.74	3.42	**0.044**	**0.11**
Il6	*N vs S*	*Group*	0.01	1	0.58	3.48	0.087	0.15
		Time		2	0.16	0.98	0.405	0 (-0.03)
		*Group*Time*		2	0.11	0.67	0.528	0 (-0.05)
	N/S vs HI	*Group*	0.09	1	16.73	17.30	**0.0002**	**0.30**
		Time		2	1.12	1.16	0.325	0.01
		*Group*Time*		2	2.35	2.43	0.102	0.07

Figure 3.34. *Tnfα* expression (hippocampus): plot of secondary analyses

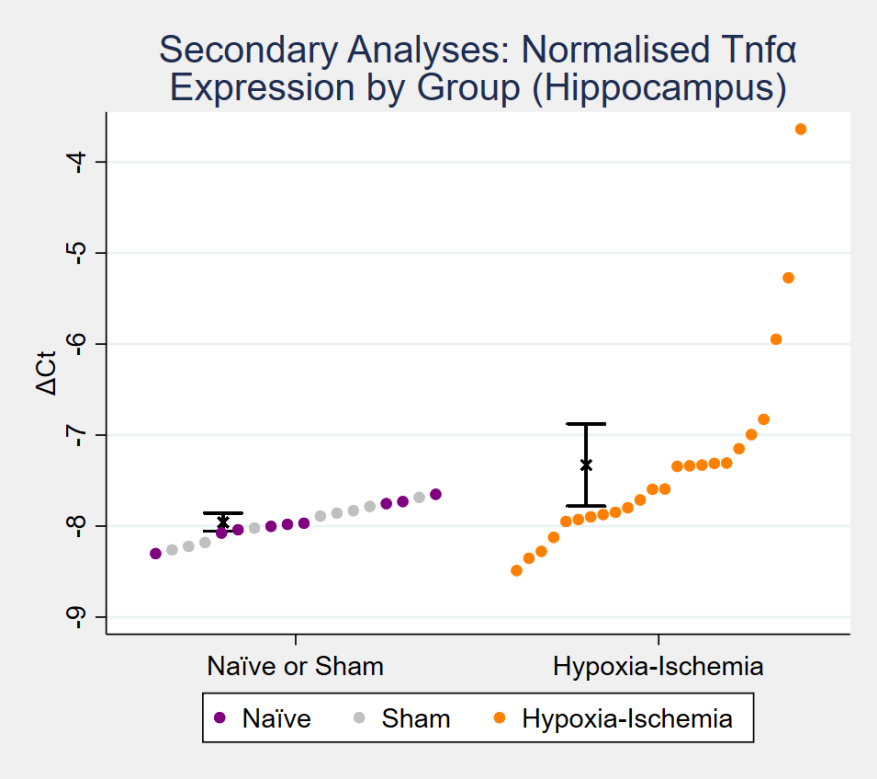


Figure 3.35.Il1β expression (hippocampus): plot of secondary analyses

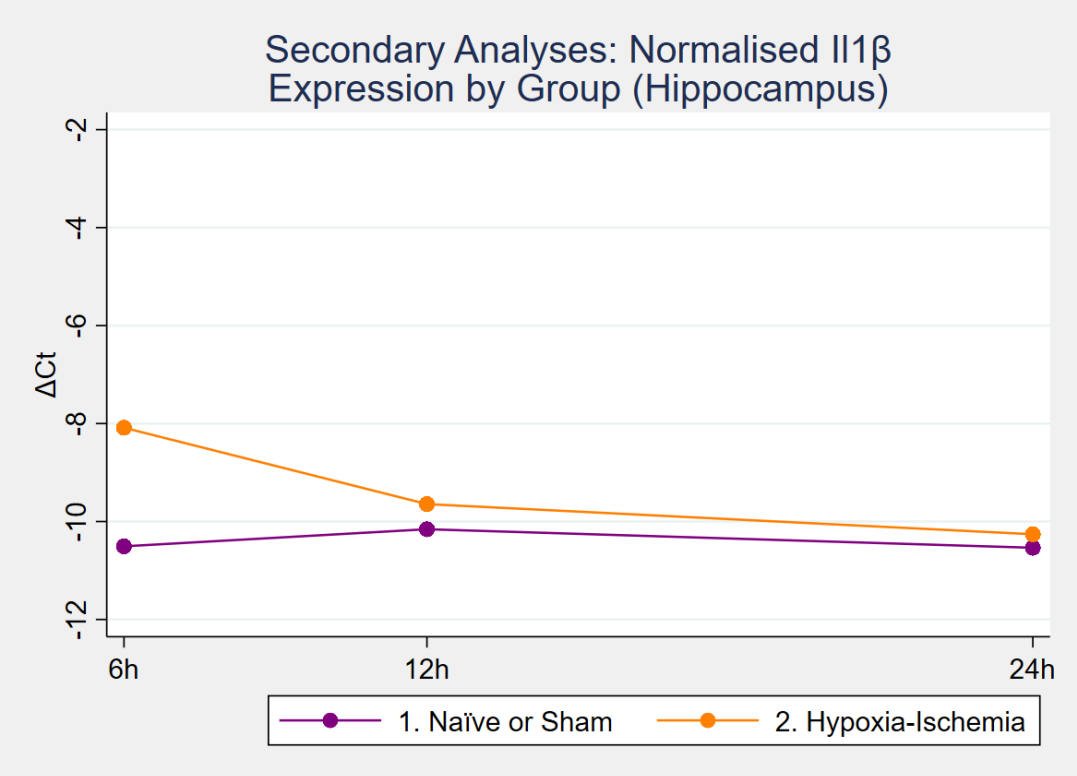
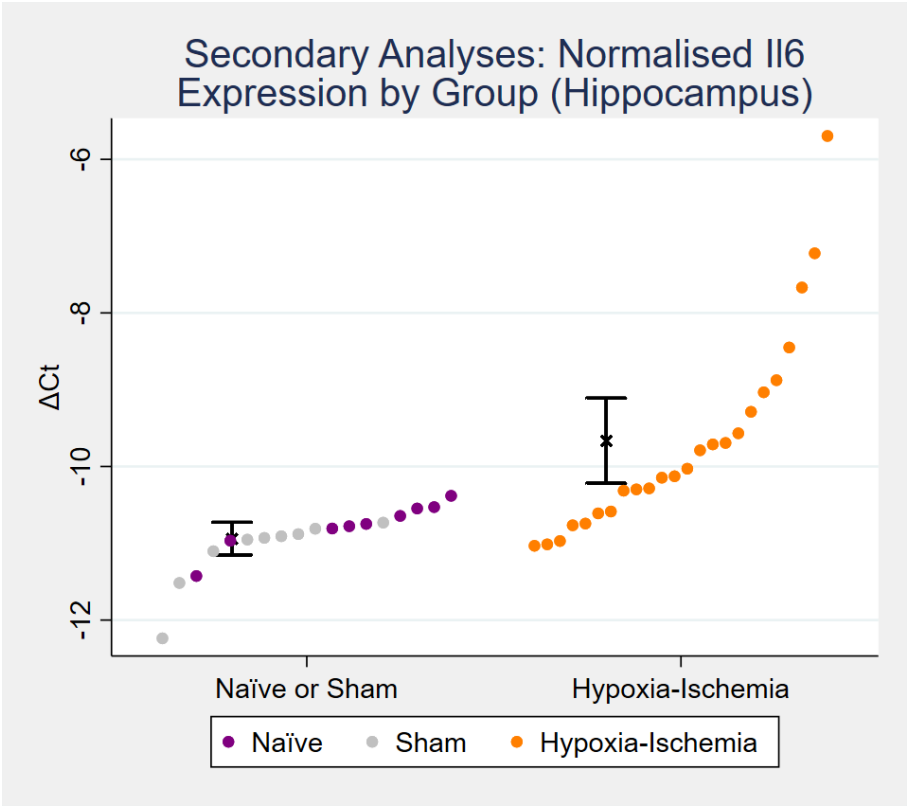


Figure 3.36. *Il6* expression (hippocampus): plot of secondary analyses



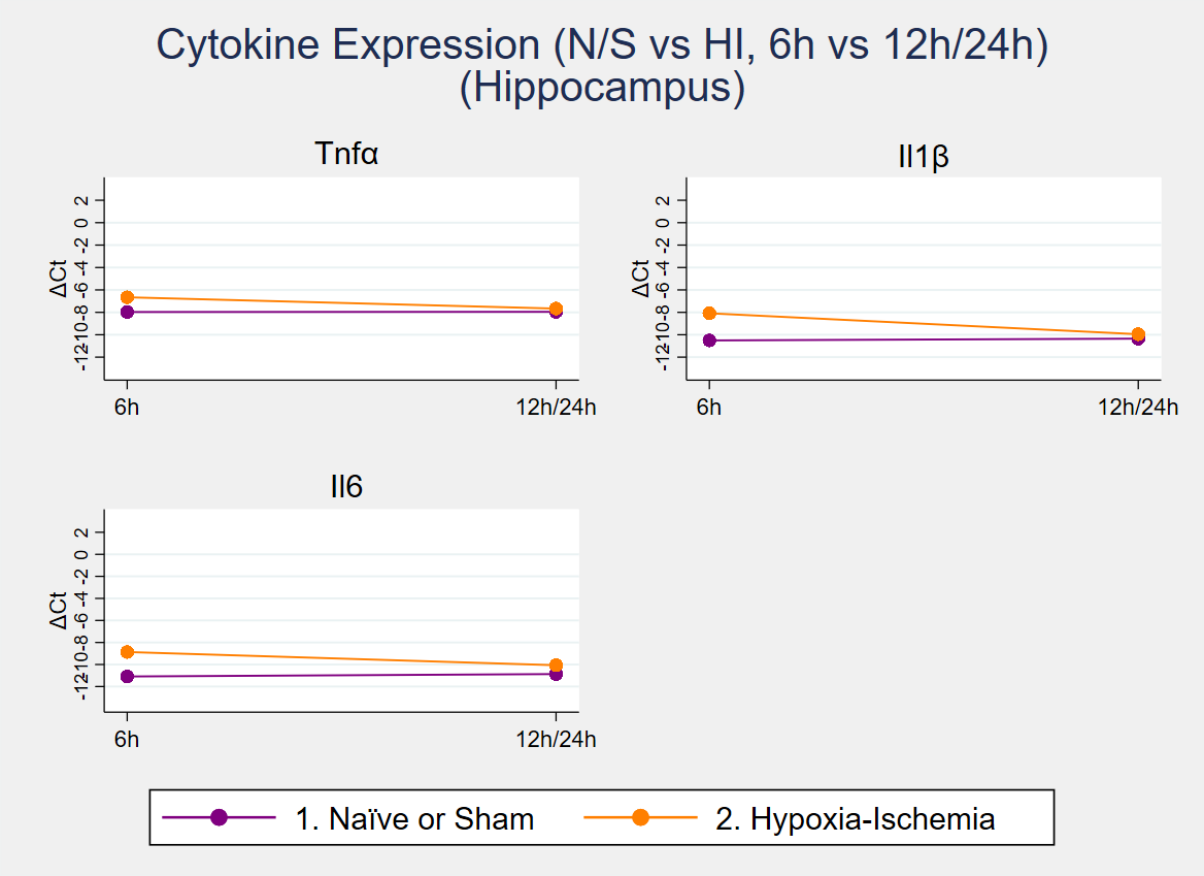
Given the non-significant trend for early upregulation of the cytokines in the 6h HI group, sensitivity analyses were carried out merging the 12h and 24h time points in an early (6h) vs late (12h + 24h) design comparing the HI group vs the control group (N/S) (Table 3.22, Figure 3.37). Evidence for the interaction effect between group and time strengthened for all cytokines, including *Tnfα* (p=0.045) with an effect size of 8%, *Il1β* (p=0.013) with an effect size of 13%, and *Il6* (p=0.031) with an effect size of 9%.

Table 3.22. Inflammation expression (hippocampus): sensitivity analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	P	Effect size
	Group (N/S vs HI)		1	5.85	10.36	0.003	0.19
Tnfα	*Time (6h vs 12h/24h)*	0.01	1	2.24	3.97	0.054	0.07
	*Group*Time*		1	2.42	4.28	**0.045**	**0.08**
	Group (N/S vs HI)		1	18.13	13.27	0.001	0.23
Il1β	*Time (6h vs 12h/24h)*	0.001	1	6.66	4.88	0.033	0.08
	*Group*Time*		1	9.39	6.87	**0.013**	**0.13**
	Group (N/S vs HI)		1	20.90	22.73	<0.00001	0.35
Il6	*Time (6h vs 12h/24h)*	0.02	1	2.18	2.37	0.132	0.03
	*Group*Time*		1	4.61	5.01	**0.031**	**0.09**

Figure 3.37. Inflammation expression (hippocampus): plots of sensitivity analyses



3.4.3.3 Glutamate transporter

Descriptive statistics are reported in Table 3.23. There were no significant changes in *Glt1* expression with group or time in primary or secondary analyses, and effect sizes were also negligible (Table 3.24**,** Figure 3.38**,** Table 3.25**,** Figure 3.39).

Table 3.23. *Glt1* (hippocampus): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; SD: standard deviation

Group	Time	n	*Glt1*	
			Mean ΔCt	SD
N	**6**	3	1.67	0.17
	12	3	1.74	0.43
	24	3	1.87	0.24
S	**6**	3	1.93	0.16
	12	3	1.76	0.28
	24	3	2.10	0.17
HI	**6**	8	1.73	0.41
	12	8	1.72	0.46
	24	8	1.85	0.25

Table 3.24. *Glt1* (hippocampus): primary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Variable	Levene's p	df	MS	F	p	Effect size	*Post hoc* Scheffe's p
Glt1	*Group (N vs S vs HI)*	0.45	2	0.10	0.83	0.445	0 (-0.01)	
	Time		2	0.13	1.09	0.348	0.01	
	*Group*Time*		4	0.02	0.15	0.963	0 (-0.1)	

Figure 3.38. *Glt1* expression (hippocampus)

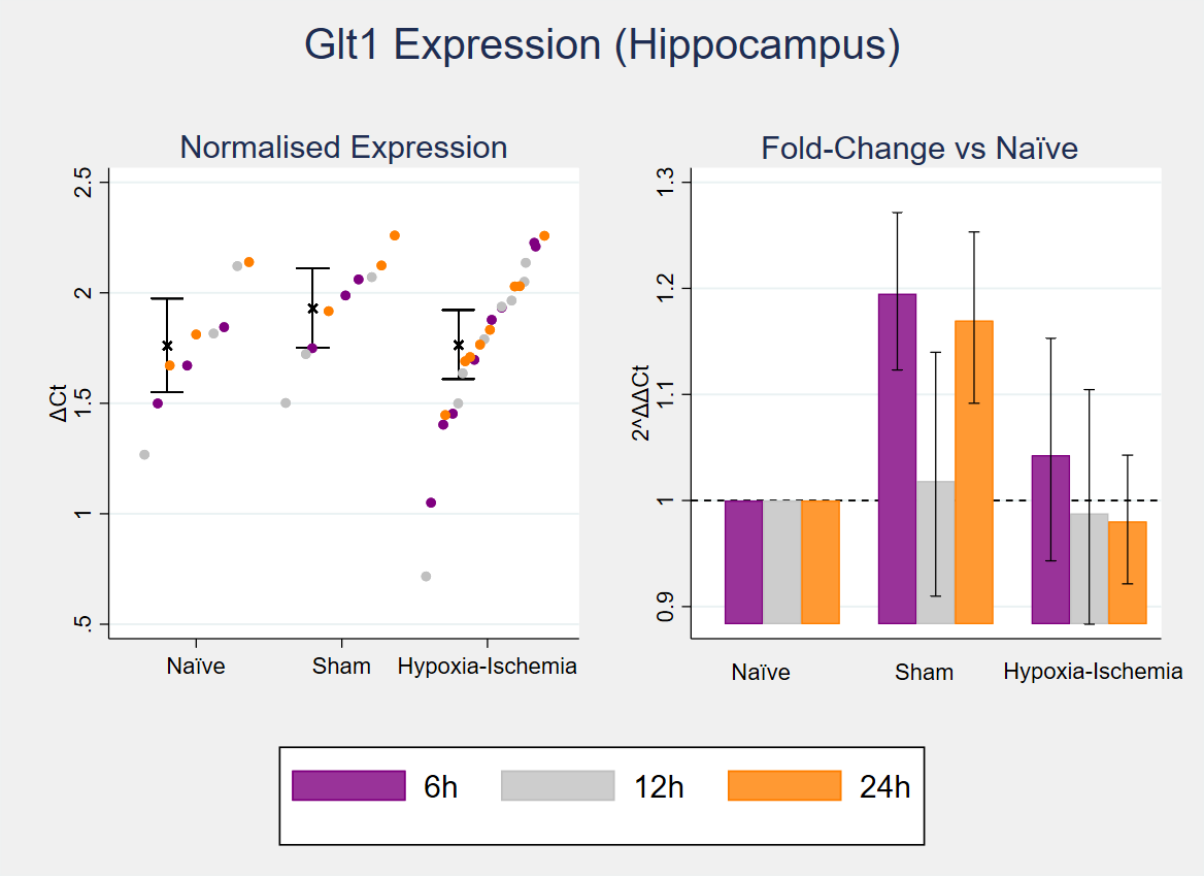
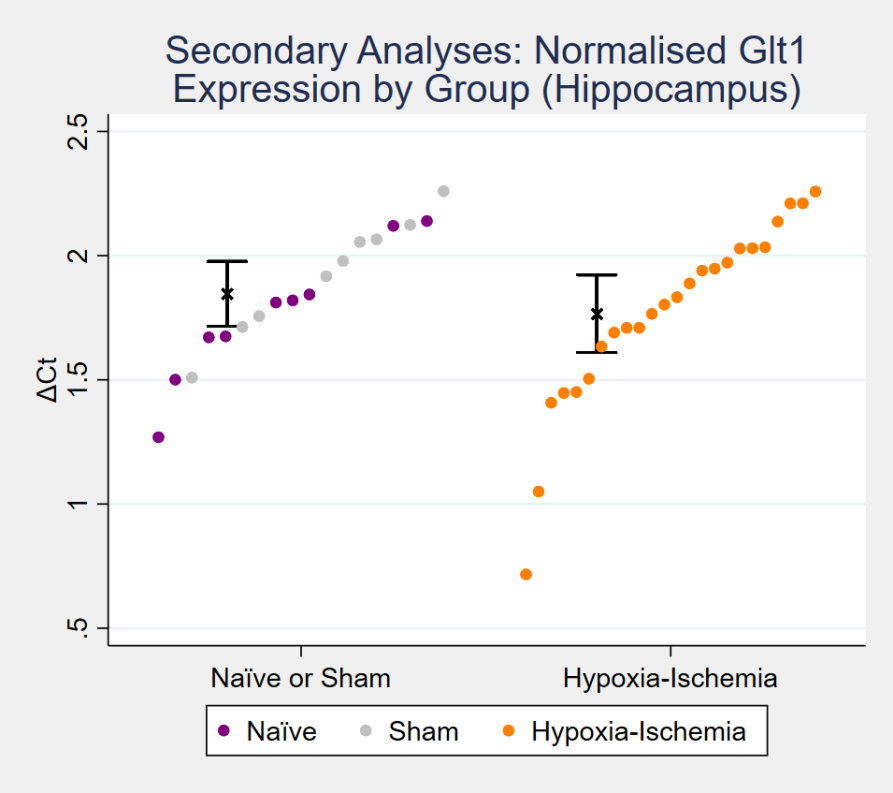


Table 3.25. *Glt1* (hippocampus): secondary analyses

df: degrees of freedom; MS: means squares; F: F ratio; N: naïve; S: sham; HI: hypoxia-ischaemia

Gene	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
Glt1	*N vs S*	*Group*	0.38	1	0.13	1.91	0.192	0.06
		Time		2	0.09	1.38	0.288	0.05
		*Group*Time*		2	0.02	0.35	0.713	0 (-0.09)
	N/S vs HI	*Group*	0.40	1	0.07	0.59	0.449	0 (-0.01)
		Time		2	0.13	1.14	0.331	0.01
		*Group*Time*		2	0.01	0.10	0.908	0 (-0.05)

Figure 3.39. *Glt1* expression (hippocampus): plot of secondary analyses

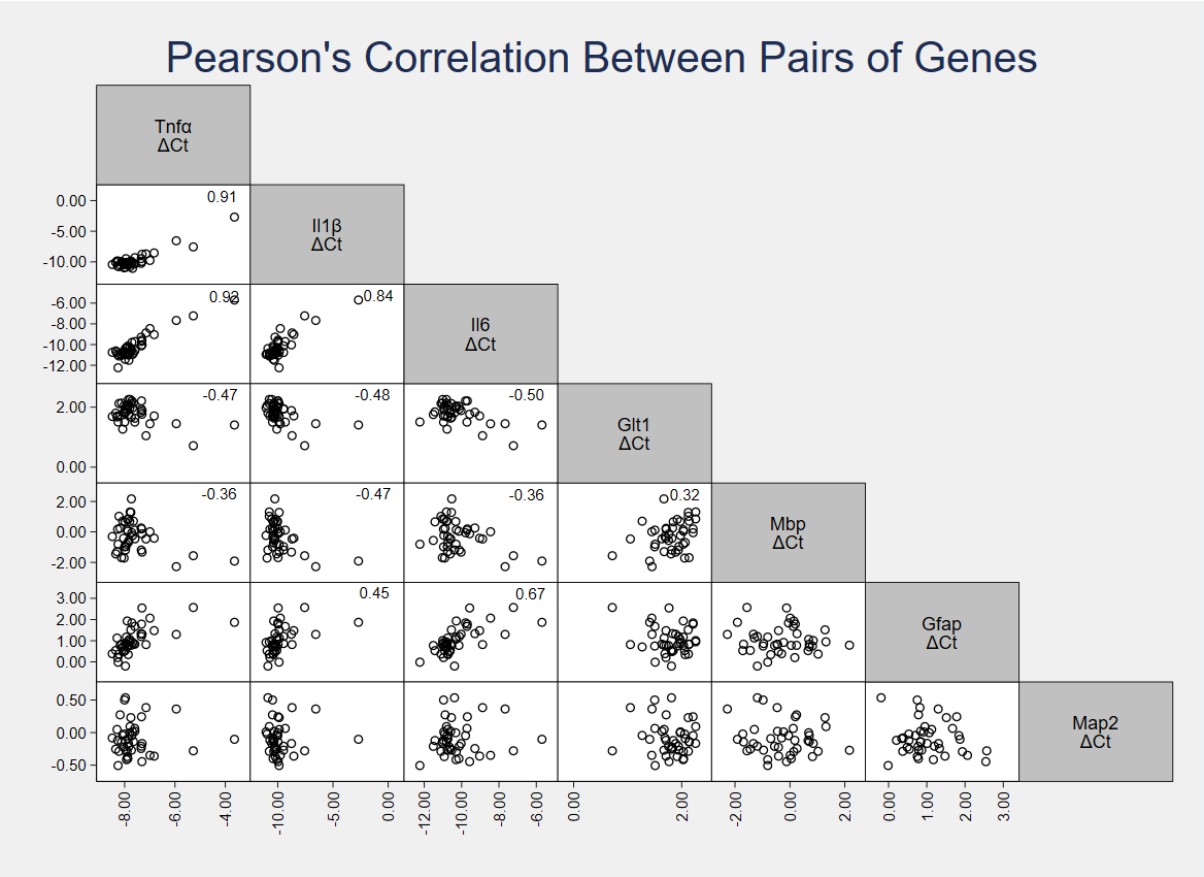


3.4.3.4 Comparison across genes

Correlations between pairs of genes were tested with pairwise Pearson's correlation coefficient and the significant coefficients ($p \leq 0.05$, not corrected for multiple comparisons) annotated on pairwise scatterplots (Figure 3.40). There was a strong positive correlation between all three cytokines (Pearson's correlation coefficients ≥0.8), so that as expression of one cytokine increased so did expression of the others. The correlation was particularly strong between *Tnfα* and both *Il1β* and *Il6* (Pearson's correlation coefficients =0.9) There was a weak negative correlation between *Glt1* and all cytokines (Pearson's correlation coefficients =-0.5). In terms of markers of injury, *Mbp* showed a weak positive correlation to *Glt1* (Pearson's correlation coefficient =0.3) and a weak negative correlation to the cytokines (Pearson's correlation coefficients ≤-0.4). *Gfap* showed a moderate positive correlation to *Il6* (Pearson's correlation coefficient = 0.7) and a weak positive correlation to *Il1β* (Pearson's correlation coefficient = 0.5). *Map2* was not correlated to any gene.

Figure 3.40. Matrix of pairwise correlations in gene expression (ΔCt) (hippocampus)

Only correlation coefficients with p≤0.05 are reported



In sensitivity analyses, the ten pups showing the most extreme normalised expression values (untransformed ΔCt) for each gene were reported in Table 3.26. "Extreme" referred to the highest expression values for *Gfap* and the cytokines, and lowest for *Mbp*, *Map2* and *Glt1*, based on current findings and the direction of change expected from previous literature. Outliers, as seen in the plots, are indicated with a star. The rats with the most extreme expression for one gene (ranked 1st-2nd) tended to be amongst those with the most extreme expression for at least 3 other genes (ranked 1st-10th).

This was seen more consistently for the cytokines and *Gfap* and less so for *Mbp*, *Map2* and *Glt1*, which showed weak or no group effects in the analyses. For example, the pup in 6h HI group with highest expression of *Tnfα* (outlier in Figure 3.31) is also the pup with highest expression of *Il1β* (outlier in Figure 3.32) and *Il6* (outlier in Figure 3.33) and is amongst the pups with highest expression of *Gfap* and lowest expression of *Glt1* (in purple). Similarly, the pup with lowest expression for *Glt1* was also the pup with highest *Gfap* expression and amongst the pups with highest cytokine expression and lowest *Mbp* expression (in green).

The pup with lowest *Mbp* expression was also amongst the pups with highest cytokine expression (outliers in Figure 3.31, Figure 3.32, Figure 3.33) and lowest *Glt1* expression (in orange). Since there was no indication that the outliers were artifacts, they were retained in the analyses.

With regard to *Glt1*, the pup with lowest *Glt1* expression (in green) was from the 12h HI group and also amongst the pups with the highest cytokine levels and *Gfap*, and lowest *Mbp*. The second in rank for *Glt1* (in yellow) was from the 6h HI group and also amongst the pups with highest cytokine levels. The third in rank for *Glt1* (red) was from the 12h naïve group and did not feature amongst the pups with most extreme expression of any of the other genes. The fourth in rank was also from the 6h HI group, also had the highest cytokine levels across the entire sample and was amongst the pups with highest level of *Gfap* (in purple). Therefore, three of the four pups with lowest *Glt1* expression were from the HI groups and also amongst the pups with the highest cytokine levels. While some interesting patterns emerge from these data, these are sensitivity analyses from a small pilot study and must be interpreted with caution.

Table 3.26. Top 10 pups ranked by most extreme gene expression for each gene (hippocampus)

"Extreme": the highest or lowest expression value within the sample, depending on the expected direction of change based on these findings and previous literature, i.e. highest to lowest expression for the cytokines and *Gfap* (▼); lowest to highest expression (▲) for *Glt1*, *Mbp* and *Map2*.

Cell numbers are rat IDs (e.g. 6H5 is the 5th of one of the 8 rats from the hypoxia-ischaemia group at 6h; N: naïve; S: sham; H: hypoxia-ischaemia). Each rat ID has been assigned one colour in the table (e.g. 6H5 is shown in orange). Outliers (see corresponding figures in the main text) are marked with *.

Mbp▲	*Gfap*▼	*Map2*▲	*Tnfα*▼	*Il1β*▼	*Il6*▼	*Glt1*▲
6H5	12H4	12S2	6H2*	6H2*	6H2*	12H4
6H2	24H2	24H2	12H4*	6H5*	12H4*	6H1
6S3	24H4	24H8	6H5*	12H4*	6H5*	12N2
12H5	24H3	12H7	6H8	6H8	24H4	6H2
12H4	6H2	24S2	24H4	6H1	6H1	24H4
12H8	6H7	6H8	6H1	6H4	6H8	6H5
6H4	6H3	24H4	6H4	12H1	24H7	6N1
6N3	12H1	24H3	24H2	6H6	24H2	12H1
6H6	12N1	24S3	6H3	12H6	6H3	12S2
24N3	6H8	12H4	6H6	24H4	12H1	12H3

3.4.4 Comparison across regions

The marker of astrocyte activation *Gfap* was strongly increased after HI in both cortex and hippocampus, with approximately similar level of evidence and effect size (cortex: p=0.001, ω^2=0.29; hippocampus: p=0.004, ω^2=0.24). In the cortex but not the hippocampus, there was a non-significant trend for higher expression in the later phase (24h) compared to the earlier phases (6h and 12h), with 3-fold higher expression in the HI group compared to the naïve group at 24h.

The marker of myelin injury *Mbp* was lost after HI in the left cortex (cortex: p=0.0001, ω^2=0.40), with secondary analyses merging naïve and sham suggesting a slight reduction in the hippocampus too (hippocampus: p=0.035; ω^2=0.09). In the hippocampus, there was a significant time effect whereby *Mbp* was lower in the early phase (6h) compared to the later phase (24h) in all groups.

There was no evidence of changes in neuronal marker *Map2* in either region. There was also no evidence of changes in *Glt1* in either region. Secondary analyses merging naïve and sham suggested that *Glt1* may be slightly reduced in the cortex after HI (cortex: p=0.036, ω^2=0.09).

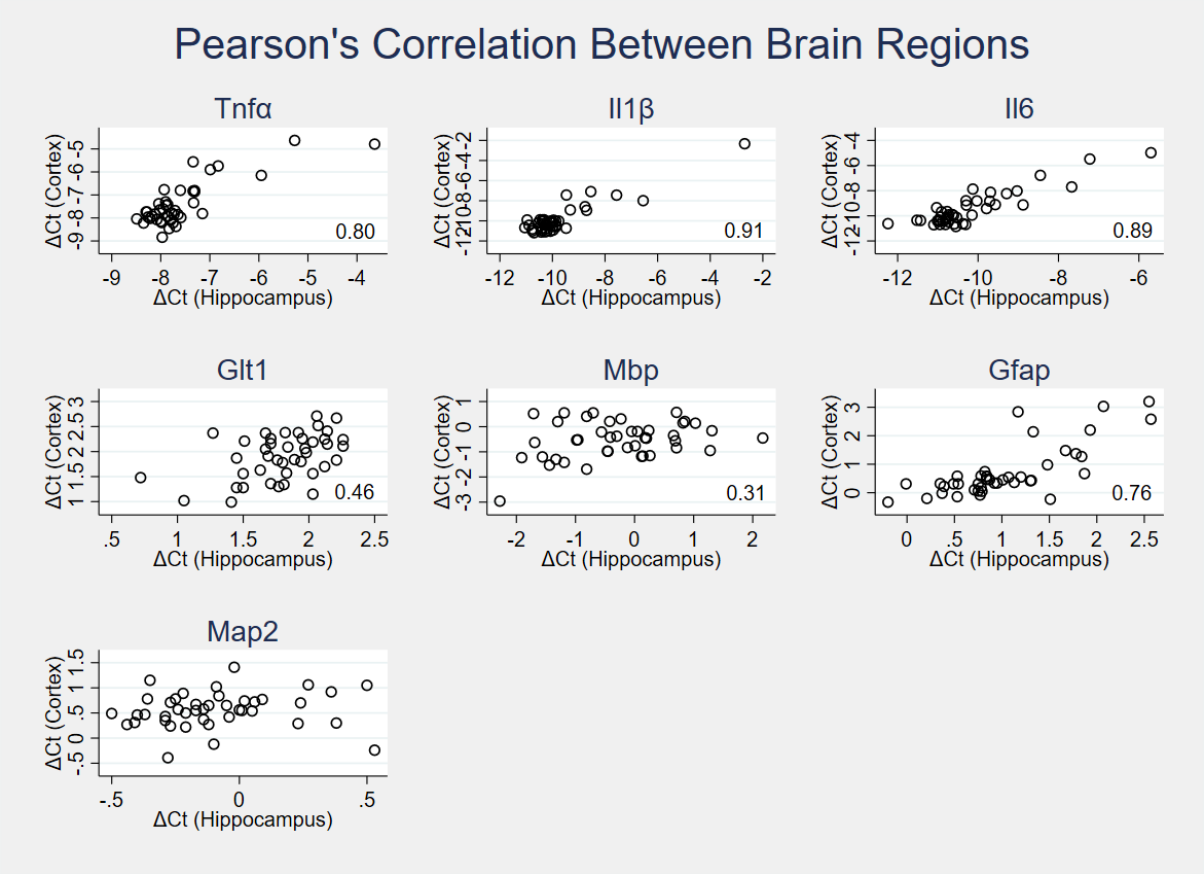
All three pro-inflammatory cytokines were elevated after HI in both brain regions. Effect size was the same in both regions for *Il1β* (cortex: p=0.030, ω^2=0.14; hippocampus: p=0.027, ω^2=0.14) and *Il6* (cortex: p=0.001, ω^2=0.29; hippocampus: p=0.001, ω^2=0.29). Effect size for *Tnfα* was twice as large in the cortex vs hippocampus (cortex: p=0.005, ω^2=0.23; hippocampus: p=0.054, ω^2=0.11), i.e. group accounted for twice as much variation in *Tnfα* expression in the cortex compared to the hippocampus. In both regions, there was a non-significant trend for higher expression in the early phases (6h) post-HI compared to the later phases (12h and 24h). In the secondary analyses assessing early (6h) vs late (12h + 24h) response in the HI group vs control group (N/S), evidence of an interaction effect with upregulation at 6h in the HI group reached significance for all cytokines in the hippocampus (*Tnfα*: p=0.045, ω^2=0.08; *Il1β*: p=0.013, ω^2=0.13; *Il6*: p=0.031, ω^2=0.09) and for *Il1β* in the cortex (p=0.013, ω^2=0.13), with a non-significant trend for *Tnfα* (p=0.069, ω^2=0.06) and *Il6* (p=0.133, ω^2=0.03) in the cortex.

To summarise findings within each brain region, there was a strong positive correlation between all three cytokines (Pearson's correlation coefficients ≥0.8), especially between *Tnfα* and *Il6* in the cortex and between *Tnfα* and both *Il1β* and *Il6* in the hippocampus (Pearson's correlation coefficients =0.9). There was also a weak negative correlation between *Glt1* and

all cytokines (Pearson's correlation coefficients ≤-0.4), with the weakest correlation seen for *Glt1* and *Tnfα* in the cortex (Pearson's correlation coefficient =-0.35). In terms of markers of injury, *Mbp* was positively correlated to *Glt1*, with stronger correlation in the cortex than in the hippocampus (Pearson's correlation coefficients =0.6 vs 0.3). *Mbp* also showed a weak negative correlation with all cytokines in both regions, with a slightly stronger correlation for *Tnfα* and *Il6* in the cortex than in the hippocampus (Pearson's correlation coefficients =-0.5 vs -0.35). *Gfap* showed a moderate positive correlation to *Il6* in both regions (Pearson's correlation coefficients ≥0.6), a moderate positive correlation to *Tnfα* but not *Il1β* in the cortex (Pearson's correlation coefficients = 0.6), and a weak positive correlation to *Il1β* but not *Tnfα* in the hippocampus (Pearson's correlation coefficient = 0.5). In the cortex, *Gfap* additionally showed a weak negative correlation to *Glt1* and *Mbp* (Pearson's correlation coefficients =-0.4). *Map2* only showed a weak positive correlation with *Glt1* in the cortex (Pearson's correlation coefficients =0.3).

Between regions (Figure 3.41), there was a strong positive correlation of all of the cytokines as well as *Gfap* (Pearson's correlation coefficients ≥0.76), so that if expression was high in the cortex it tended to be high in the hippocampus as well. Thus, pups with particularly high expression in the cortex tended to correspond with pups with particularly high expression in the hippocampus (and generally had very low/high levels of at least three other genes compared to the other pups). There was a weak positive correlation between left cortex and left hippocampus for *Glt1* (Pearson's correlation coefficients =0.46) and *Mbp* (Pearson's correlation coefficients =0.31), i.e. as expression of *Glt1* and *Mbp* in the cortex decreased, there was more variability in the hippocampus. There was no significant correlation between brain regions for *Map2*.

Figure 3.41. Pairwise correlations (between bran regions)



After Benjamini-Hochberg False Discovery Rate correction, 9 findings from the primary analyses survived multiple comparison adjustment, with an accepted false discovery rate of 7% (i.e. one gene in one of the two brain regions amongst 14 tests) (Table 3.27). The findings surviving FDR adjustment (shaded) included *Mbp* in the cortex, as well as *Gfap* and all cytokines in both brain regions.

Table 3.27. Gene expression: findings surviving Benjamini-Hochberg False Discovery Rate (7%) correction (shaded)

Gene	Region	p-value for Group Effect (Primary Analyses)	Rank	Benjamini-Hochberg critical value
Mbp	Cortex	0.0001	1	0.007
Gfap	Cortex	0.001	2	0.014
Il6	Cortex	0.001	3	0.021
Il6	Hippocampus	0.001	4	0.029
Gfap	Hippocampus	0.004	5	0.036
Tnfα	Cortex	0.005	6	0.043
Il1β	Cortex	0.030	7	0.050
Il1β	Hippocampus	0.027	8	0.057
Tnfα	Hippocampus	0.054	9	0.064
Mbp	Hippocampus	0.093	10	0.071
Glt1	Cortex	0.098	11	0.079
Map2	Hippocampus	0.138	12	0.086
Map2	Cortex	0.320	13	0.093
Glt1	Hippocampus	0.445	14	0.100

3.5 Discussion

This exploratory study used the Rice Vannucci model to assess whether acute HI alters transcription of three key pro-inflammatory cytokines (*Tnfα*, *Il1β*, *Il6*) and the glutamate transporter (*Glt1*) in two of the most vulnerable brain regions, the cortex and hippocampus. mRNA levels of the candidate genes were assessed with qPCR and normalised to two housekeeping genes (*Hprt*, *B2m*) to account for confounding differences in the starting amount of template. Since brain injury and survival could not be directly assessed in this study, gene expression of established markers of neuronal injury (*Map2*), myelin injury (*Mbp*) and astrogliosis (*Gfap*) was also assessed. Transcription of the neuronal marker was not affected, although post-translational regulation was not assessed and nor were other neuronal markers. Changes in the other markers support clinical relevance of the model, since they signal some of the most common neuropathological features seen in HIE, i.e. early onset of astrogliosis and myelin injury. All three cytokines were upregulated following HI in both brain regions, with a trend for peak upregulation at 6h, providing evidence of an early neuroinflammatory response. There was no evidence of significant changes for the glutamate transporter, other than suggestive evidence of lower expression in the HI group at the secondary analysis stage merging naïve and sham groups. This may be an artefact or flag an effect in the astrocyte compartment which the study is underpowered to detect with current sample size and bulk tissue.

3.5.1 Hypoxia-ischaemia induces astrogliosis and myelin injury in both regions

Astrocytes can be directly injured and die via necrosis in the infarct area following HI. Additionally, surviving astrocytes nearby the injured tissue ('the penumbra area' or border zone) can become reactive due to the locally rising levels of danger associated molecular patterns, cytokines and reactive oxygen species released by injured neurons and glial cells (804, 1215). During astrogliosis, astrocytes become hypertrophic, proliferate at an increased rate and alter their gene expression profiles (1216). One of the hallmarks of astrogliosis is rapid accumulation of intermediate filament protein GFAP (1217, 1218). Astrogliosis with GFAP staining has been shown in a model of severe HIE in nonhuman primates (1219). In the rodent brain, HI causes rapid upregulation of GFAP mRNA and protein in the cortex, hippocampus, striatum, thalamus, and corpus callosum, starting at 12h-72h and gradually increasing in the first two postnatal weeks (483, 1220-1222). Accordingly, we found that *Gfap* mRNA was upregulated rapidly after HI in both cortex and hippocampus, with a trend for peak upregulation at 24h which was especially obvious in the cortex. Of interest, accumulation of GFAP at the border zone corresponds to areas with loss of MAP2 protein and impaired neuronal growth (1221, 1223, 1224). In a similar rat model, *Gfap* accumulation switched location from the border zone at 12-72h to the infarct core, where it persisted until adolescence (1221). This highlights a potential chronic component to astrogliosis and suggests *Gfap* upregulation would have likely continued past 24h.

MBP is essential for the formation and maintenance of the myelin sheath, and the abundant transcripts provide an easily measurable marker of the amount of myelin and oligodendrial injury (1225, 1226). In rodents, loss of *Mbp* mRNA and oligodendrocyte death are observed as early as 3 hours post-HI and are obvious by 24-96h in the subcortical white matter, corpus callosum and deep grey matter (675, 1227-1229). Loss is sustained if injury is severe or can recover 2 weeks after HI, likely due to migration of new oligodendrocytes from the subventricular zone or maturation of spared pre-oligodendrocytes (1228, 1230-1232). Of note, therapeutic hypothermia protects against white matter injury measured as MBP loss (1233-1235). In this pilot study, HI caused *Mbp* loss in the ipsilateral cortex, signalling the onset of myelin and oligodendrial injury. This loss was detectable from the earliest 6h time point to latest 24h time point. In the hippocampus, *Mbp* was lower at 6h vs 24h regardless of experimental group. The weaker evidence here was likely due to high variability in the naïve group coupled with small sample size, and a trend for *Mbp* loss following HI was present.

Importantly, baseline *Mbp* levels are still low in both white matter and hippocampus at P7, increasing from the second postnatal week (1228).

MAP2 is a structural protein expressed in dendrites and an established marker of HI neuronal injury, with transient protein loss observed 1-72h post-HI in the hippocampus, cortex, and thalamus of newborn rats and piglets (684, 1236-1243). Loss of MAP2 correlates with histological evidence of neuronal injury, with complete loss in the ischemic core/infarct area and partial loss in the border area, detectable within the first 12h (684, 1240). Moreover, loss of MAP2 precedes microglial activation in the cortex, striatum and hippocampus of the newborn rat, whereas delayed loss coincides with delayed microglial activation in the thalamus (1244). MAP2 loss is also seen in the cortex and hippocampus of infants younger than 1 year of age with HI injuries (1245). Therefore, MAP2 protein appears to be an early and sensitive marker of the distribution, time course and severity of neuronal injury after HI. In this pilot study, we did not examine MAP2 protein or neuronal ischemic injury directly by immunohistochemistry, and we found no evidence of changes in *Map2* mRNA. Rodent studies focusing on *Map2* mRNA instead of protein are rarer, but nonetheless include evidence of loss after HI and correlation with neuronal injury in adults and after systemic inflammation in newborns (484, 1246).

The lack of changes in *Map2* mRNA levels may have arisen through technical error, with selective amplification of certain alternative splicing isoforms. However, qPCR primers for *Map2* were designed to span exon 3 and 4, which belong to all known isoforms, and should therefore capture total mRNA expression. Alternatively, mRNA and protein levels may be at least in part uncoupled. This explanation is supported by the finding that MAP2 protein levels increase in the developing mouse from birth to P21, however mRNA levels remain more constant (1247). While qPCR detects the levels of mature mRNA transcripts (spliced, polyadenylated and capped), regulatory processes continue after transcription, and include transport to the cytoplasm, degradation, localisation to different cellular compartments and translation (1248). Protein assays could be used to assess MAP2 protein levels, however the most appropriate next step would be assessing mRNA levels of another established neuronal injury marker, *NeuN*. This marker is localised in and around the nucleus of most neurons and has been previously used in this and other relevant animal models, including in combination with MAP2 (1202, 1227, 1249-1251).

In conclusion, although brain tissue loss was not directly measured in this pilot study due to the early time points for sacrifice, the rat model appears clinically relevant since it reproduces some of the most common neuropathological features seen in HIE, i.e. early onset of astrogliosis and myelin injury.

3.5.2 Hypoxia-ischaemia induces an early neuroinflammatory response in both regions

Transcription of three key pro-inflammatory cytokines (*Tnfα*, *Il1β*, *Il6*) was increased in both cortex and hippocampus following HI, with a trend for peak upregulation at 6h. This finding aligns with previous evidence of upregulation in similar rat models in the ipsilateral vs contralateral hemispheres starting as early as the first hour, peaking at 3-12h and returning to baseline by 24h (555, 1221, 1252). The finding also aligns with evidence of increased *Tnfα* and *Il1β* mRNA levels 3h post-HI in the ipsilateral hemisphere compared to sham rats, with simultaneous infiltration of peripheral neutrophils and microglial activation. A switch to anti-inflammatory mechanisms was seen after 24h, with *Tnfα* levels remaining elevated at 24h (1253). In another study of combined inflammatory and HI insults, the group exposed to HI only (plus vehicle NaCl injection) showed only a mild upregulation of *Il6* in the hippocampus but not the cortex, and no difference in *Il1β* in either region (*Tnfα* not investigated); however, the only time point included was at 24h, which may have led to missing any changes in gene expression at earlier time points (1254). The current study features three time points in the early phases of injury and adds a regional dimension rather than analysing hemispheres in bulk, with detection of a transient inflammatory response in two of the most vulnerable brain regions. These cytokines are known to interact and stimulate each other and are often expressed concurrently (1255, 1256). This was reflected by the correlation between cytokines within each region and across regions. Importantly, previous evidence exists that an increase in IL1β and IL6 protein levels and activity accompanies the increase in mRNA in relevant rodent models (555, 558). Protein assays should be employed in future studies to assess the correlation between mRNA and proteins over time and obtain a more complete picture of cytokine regulation following HI. While rapid *de novo* transcription of cytokine mRNA (e.g. via activation of NFκB and STAT3) is key in mediating the acute phases of inflammation, post-transcriptional regulation may be important for resolving inflammation after the acute phase, for example by degradation of mRNA transcripts or storage for translation at a later stage (1257-1259).

In terms of fold-change, the magnitude of upregulation in the HI vs naïve groups was highest for *Il1β* in the cortex (6-fold), followed by *Il1β* and *Il6* in both regions (4- to 5-fold) and

finally *Tnfα* in both regions (3-fold). The values of the fold-changes need to be interpreted with caution since this was a small pilot study with low power and, as expected from the model, variability was relatively high for the HI group. Compared to fold-changes relative to the age-matched control groups, standardised effect sizes (ω^2) take into account variability within the 3 x 3 design. Within this framework, the larger effect size was found for *Il6* in both regions and *Tnfα* in the cortex (group effect accounting for 20-30% variation in gene expression) followed by *Il1β* in both regions and *Tnfα* in the hippocampus (group effect accounting for 10-15% variation in gene expression). The most appropriate way to estimate effect sizes more precisely will be via a follow-up experiment with larger sample sizes.

Low power reduces the chance of detecting a true effect but also the likelihood that a detected effect is true (1260). This is likely to lead not only to missing important discoveries, but also to overestimate true effect sizes and poor reproducibility. Exploratory studies with particularly low power, such as this one, can only detect effects that are large and may overestimate smaller effects when detected. This is because statistical significance may not be reached unless effects that are by chance larger than the true effect size are detected. This leads to inflated effects in the initial studies ("winner's curse") which are followed by smaller effects in replication studies, as findings converge towards the true effect size. For this reason, replication studies should feature larger sample sizes. This is a high priority in light of recent evidence pointing to particularly severe power issues specific to this rat model. A study recently merged samples from multiple smaller studies with this model reaching a sample size of over 600 for the first time (1199). In this larger analysis, a bimodal distribution of injury (i.e. brain area loss or pathology scores) was observed. This suggests that there are two distinct population of rats with differences in underlying initial characteristics, for example in blood flow, which may contribute to differences in brain injury (1203, 1261). A bimodal distribution was indeed observed for the cytokines and *Gfap*, indicating that most rats had either high or low expression. The observations from the large study led to the suggestion that non-parametric analyses should be used in studies with this model. Moreover, it was acknowledged that most existing studies are markedly underpowered to detect true effects and much larger sample sizes are required to produce robust findings (1199). Despite the issue of low power, biological relevance in this pilot study is supported by the consistency in direction of effect and temporal profiles, the correlation within and between brain regions and the agreement with the existing literature.

In terms of time points, there was a non-significant trend for peak upregulation at 6h for all three cytokines; at subsequent time points, *Il1β* levels went back to baseline in both regions, alongside *Tnfα* in the hippocampus, whereas they were reduced but still 1.5- to 2-fold higher for *Il6* in both regions and *Tnfα* in the cortex. Based on previous evidence, upregulation of these cytokines is likely to have started earlier than our first time point at 6h (555, 1221, 1252). Increased power may be achieved in future work by considering two rather than three time points (e.g. 6h vs 24h, or 3h vs 6h). Assessing gene expression at earlier time points may provide more definitive evidence of the peak time of the early increase in transcription within this model; on the other hand, assessing later time points may provide insights into which cytokines tends to have a more sustained upregulation or biphasic patterns of expression. This has been shown for some inflammatory genes (including *Il6* and *NFκB*) in HIE, suggesting that they might switch roles from mediators of injury to mediators of repair in the secondary phases (1262-1265). Exploring interaction with hypothermia treatment will also be of clinical interest, especially since evidence is emerging that it may alter cytokine expression and promote repair roles (1265-1267).

3.5.3 Hypoxia-ischaemia may suppress Glt1 in the cortex

In this pilot study, there was no significant evidence of any changes in *Glt1* expression in either cortex or hippocampus in the primary analyses. There was, however, very weak non-significant evidence that expression in the cortex may be lower in the HI group vs sham group. When naïve and sham were merged, evidence suggesting that *Glt1* may be lower in the HI group was stronger. It is not possible to know at this stage whether this is an artefact of data manipulation in the secondary analyses or a true biological effect which the study is underpowered to detect. Moreover, should this be a true biological effect, it is not currently possible to distinguish whether isoflurane upregulated *Glt1*, HI suppressed it or both.

The low power may be due to both low sample size and use of bulk tissue. The latter may be an issue specifically for *Glt1*, which is expected to be expressed mainly by astrocytes at this gestational age. Transcriptional and DNA methylation changes may therefore be detectable only after isolating astrocytes. Moreover, differences in gene expression between left and right hemispheres have been observed empirically even in naïve brains (1200). Hence, it is possible that within-rat variability may have concealed between-rat variability. Ideally, future studies will assess both hemispheres and generate a ratio of gene expression for each rat including information about both brain hemispheres. Based on the existing *in vitro* and *in vivo* evidence of dysregulation of EAAT2/GLT1 following hypoxia or HI, the glutamate

transporter remains worthy of further attention (see 5.2.2). Moreover, transport activity should be assessed alongside expression since both mechanisms can contribute to failure of the glutamate transport system, and drug compounds exist that improve both (743).

Importantly, *Glt1* transcription may be affected by HI but not within these experimental conditions. For example, transcription of *Glt1* may change after 24h post-HI, or it may change with more severe HI. The HI insult was sufficient to trigger the onset of astrogliosis and myelin injury, flagged by upregulation of *Gfap* and loss of *Mbp* respectively. Nonetheless, unlike previous studies, there was no evidence that HI caused significant neuronal injury in the first 24h. The lack of transcriptional changes in *Map2* does not rule out changes at the protein level, and indeed transcription and translation of MAP2 have been shown to be at least in part uncoupled. However, another neuronal marker (e.g. *NeuN*) should be assessed to better assess the severity of the effects of HI on neuronal integrity. *Glt1* dysregulation may also be detectable at a later developmental stage (see 5.2.2).
Any future studies should consider that the effects of isoflurane anaesthesia on *Glt1* expression in the P7 brain are not known. Isoflurane affects neuronal glutamate signalling *in vitro* and in adult rats, though changes in astrocytic expression have not been found (1268-1277). Merging naïve and sham data in the secondary analyses assumes no effect, however there is not currently enough evidence to suggest whether this is appropriate. Careful examination of the sham group should always be included in future studies.

3.5.4 Methodological refinement for future studies

This study was limited by the use of bulk tissue and the inability to directly demonstrate the cellular sources of these cytokines. Future studies could sort and isolate relevant cell types, e.g. microglia and astrocytes, by fluorescent-activated (FACS) or magnetic-activated (MACS) cell sorting, before assessing gene expression and DNA methylation. While FACS is the gold standard for cell separation at high purity, it is time consuming and requires expensive specialist equipment not required by MACS (1278). Both techniques have been used in relevant animal models of newborn brain injuries (1254, 1279).

There was no evidence that the housekeeping genes were affected by HI in this pilot study. Indeed, previous studies have reported stability of *Hprt* and *B2m* in different cell types following hypoxia *in vitro* (1280-1283). Choosing appropriate reference genes is essential since normalising to genes that are affected by the insult can lead to false positives. Conversely, the effect could be masked if the reference gene is expressed by a different cell

type than the gene of interest and the two cell types are differentially lost due to the insult. Software exist, such as GeNorm or Normfinder, that assist with identification of the most suitable housekeeping gene for certain experimental conditions. A study recently used this software to identify reference genes specifically in the cortex of the P7 rat exposed to HI. The study found that *Hprt* was the best candidate at 0h and *B2m* at 3h post-HI, with other genes being more suitable at 12h (e.g. *Pgk1*, *Ppia*, *Rplpo*) (1284). A study recently highlighted the impact of reference genes choice specifically in the neonatal rat brain cortex after intrauterine HI (1285). 5 candidate reference genes were ranked using different methods/tools (e.g. GeNorm, ΔCt method). Different methods not only proposed different candidates but also generated large differences in quantification of the gene of interest. A similar finding comes from studies in hypoxic cancer cell lines (1286), highlighting the need to validate reference genes separately for each experimental paradigm. A future study should empirically measure expression of a panel of candidate reference genes prior to the experiment to select the most appropriate genes in this Rice Vannucci model.

Finally, differences in qPCR efficiency between reference and target genes can distort differences in expression. The $2^{-\Delta\Delta CT}$ method assumes 100% qPCR efficiency however this should be experimentally assessed in a larger study. This can be done after assay optimisation by performing a series of cDNA dilutions and inferring efficiency from the observed expression values (1287, 1288).

3.5.5 Strengths and limitations

A main advantage of this study is the use of an established rat model, which has supported and enabled the translational journey of therapeutic hypothermia from bench to bedside (702, 1193, 1202, 1289, 1290). This modified Rice Vannucci model is currently being used within our research group for studies of hypothermia treatment (1199, 1202), and this pilot study provides information which is directly relevant. Nonetheless, this is a small pilot study with limited power and results must be interpreted with caution. Follow-up analyses collecting new data in larger samples are needed to address the chance of both false negatives and false positives, as well as to better estimate effect sizes.

The study provides a good overview of the early changes occurring in the first 24h, however it did not include assessment after 24h, e.g. a 72h time point may have been particularly useful for the glutamate transporter. Moreover, the study did not include histological analyses of brain injury, data on survival or behavioural assessments. The study focused on two of the

most vulnerable brain regions (cortex and the hippocampus), however potentially important gene expression changes in other regions vulnerable to injury (e.g. thalamus) were not evaluated. Gene expression was not quantified in the right hemisphere although differences in gene expression may occur between hemispheres even in naïve rats. Gene expression analysis was limited to quantification of mRNA (and DNA methylation) and did not include protein assays. The study did not evaluate cellular sources and targets of the genes assessed. Expression of four inflammatory and glutamatergic genes was assessed in this candidate gene exploratory study, however many other genes, some of which unsuspected, are likely to be involved in HI injury.

3.6 Conclusion

While brain injury and survival could not be directly measured in this pilot study, validity of the model was supported by evidence of early changes (within 24h) in *Gfap* in both cortex and hippocampus and *Mbp* in the cortex, signalling onset of astrogliosis and myelin injury. No changes in neuronal marker *Map2* were detected in either region, however it is not known whether protein was lost post-transcriptionally or if other neuronal markers were altered in the 24h post-HI. Another marker of neuronal injury (e.g. *NeuN*) should be assessed in future work, alongside markers of microglial activation (e.g. *Iba1*, *Cox2*) and apoptosis (e.g. *Casp3*).

The main finding of this study was that an acute HI insult causes an early inflammatory response in two of the most vulnerable brain regions in newborns with HIE. Specifically, transcription of three key pro-inflammatory cytokines (*Tnfα*, *Il1β*, *Il6*) was upregulated in the ipsilateral cortex and hippocampus, with a trend for early upregulation at 6h post-HI. All three cytokines were positively correlated across brain regions and to each other within each brain region, suggesting a synergistic co-regulation within the inflammatory response. The findings align with previous evidence of an early inflammatory response induced by acute HI (516).

There was no significant evidence of transcriptional changes in *Glt1* in either region, although there was a non-significant trend for differences between sham and HI. In secondary analyses, there was suggestive evidence of suppression in the HI cortex compared to a control group merging naïve and sham rats. Should this be a true biological effect, it is not known whether isoflurane upregulated *Glt1*, HI suppressed it or both. To assess this, a follow-up

study should be set up with larger sample size and ideally assessing *Glt1* regulation in isolated astrocytes.

4 Promoter DNA methylation at the glutamate transporter in a rat model of hypoxic-ischemic brain injury at term

4.1 Introduction

In the next part of the study, DNA methylation was assessed as an epigenetic mechanism potentially mediating the observed transcriptional changes. Importantly, it is worthwhile measuring DNA methylation changes even in the absence of obvious transcriptional changes since they could precede latent effects which may have been measurable after 24h. Due to funding and time limitations, DNA methylation analysis focused on the cortex and blood, and on *Glt1* and *Tnfα* in terms of candidate genes, based on previous evidence of mutual interaction (see 1.5.3.3). Unfortunately, DNA methylation analyses could not be carried out for *Tnfα* due to COVID-19-related disruptions at the end of the PhD.

DNA methylation of candidate promoter regions can be explored by bisulfite conversion of DNA followed by pyrosequencing. This technique allows accurate sequencing of short sequences (e.g. up to 60-80 bp), posing the requirement for careful selection of candidate regions. Three regions were selected for *Glt1* based on published evidence: a region within the classic CpG island near the transcription start site (TSS), a proximal region, and a distal shore region outside the CpG island (Figure 4.1).

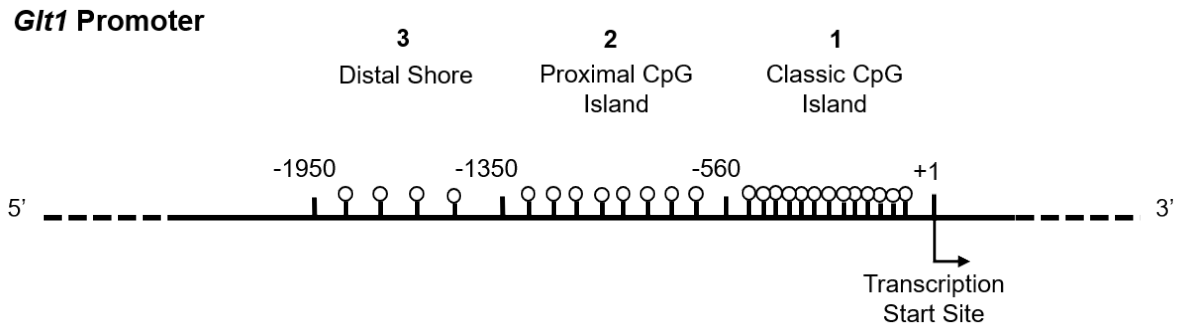
The rat *Glt1* promoter has a classic CpG island in the 5' UTR spanning approximately 560 bp upstream of the transcription start site (TSS) (1 in Figure 4.1) (1207-1209). Within the CpG island, a region was chosen that is homologous to the region overlapping the two EAAT2 -200 (C>A) and -181 (A>C) SNPs associated with adverse neurodevelopmental outcomes in humans (911). Neither SNP corresponds to a CpG site in humans, however SNPs can still affect methylation by altering binding of chromatin remodelling proteins (931, 932). For -200, the major allele is C in both humans and rats; for -181, the major allele is A in humans and C in rats, where it is also a CpG site, as identified from aligning the human and rat genomes with the Ensembl tool (human genome assembly GRCh37 with ENCODE annotations on DNA methylation, rat genome assembly Rrnor_5.0) (1291). Generally speaking, genetic variation cannot be extensively studied in inbred lab animals with limited genetic variability. However, it is still of interest to assess whether this region becomes

differentially methylated in rats following HI, since it can be hypothesised that these changes may interact with genetic influences and modify risk of injury in newborns.

The second candidate region is a proximal promoter region approximately 1350 bp upstream of the TSS (2 in Figure 4.1). This region contains one of the CpG sites (cg21163960) identified as differentially methylated in a hypothesis-free epigenome-wide association study (EWAS) comparing methylation in saliva of very preterm and healthy term newborns (1292). Multiple CpG sites in this region were hypomethylated in preterm newborns, however pyrosequencing imposes a limit on sequence length and the region overlapping this particular CpG site was chosen because it is conserved in rats.

The third candidate region is a distal shore approximately 1950 bp upstream of the TSS (3 in Figure 4.1). This sequence is upstream of the promoter CpG island, in a genomic region with lower GC content. It is more responsive to epigenetic changes including DNA methylation, responsivity which confers this region enhancer activity in reporter gene assays (1207). Differences in shore methylation in astrocytes of the cortex and cerebellum correlate with regional differences in baseline expression and in response to drugs (1207, 1208).

Figure 4.1. Diagram of the candidate promoter regions in *Glt1* chosen for DNA methylation analysis by bisulfite conversion followed by pyrosequencing



A region in the promoter of the *Peg3* gene was also included as an internal control for the bisulfite conversion and pyrosequencing methodology. *Peg3* is an imprinted gene encoding a zinc finger protein, with DNA methylation permanently silencing the maternal allele, resulting in expression of the paternal allele only (1293, 1294). As such, *Peg3* is expected to be ~40-60% methylated (0% on one chromosome and 100% on the other chromosome in all cells), and positive evidence of this would indicate the ability to effectively measure methylation within each batch of bisulfite converted DNA (bcDNA).

4.2 Hypothesis

The hypotheses of this pilot study are that:

1) acute hypoxia-ischaemia causes changes in mRNA transcription of the main glutamate transporter (*Glt1*) and three key pro-inflammatory cytokines (*Tnfα, Il1β*, *Il6*) in the term-equivalent rat brain within the first 24 hours (chapter 3)

2) any observed or latent transcriptional changes in *Glt1* and *Tnfα* in the brain are at least in part mediated by DNA methylation changes in the respective gene promoters (this chapter)

3) DNA methylation of *Glt1* and *Tnfα* in the brain correlates with DNA methylation in blood, which may be a clinically useful peripheral biomarker of injury (this chapter)

4.3 Materials and methods

4.3.1 Study design

See chapter 3.

4.3.2 Rice Vannucci rat model of moderate hypoxic-ischemic brain injury at term

See chapter 3

4.3.3 Downstream molecular analyses

Briefly, genomic DNA and mRNA were simultaneously extracted from the ipsilateral cortices and hippocampi (protocol 1). Brain DNA was further cleaned (protocol 5) using the same column kit for blood DNA extraction and clean-up (protocol 6). DNA from both brain and blood was subjected to bisulfite conversion (protocol 7), which chemically alters DNA allowing differentiation between methylated and unmethylated cytosines at CpG islands. The candidate promoter regions in the bisulfite converted DNA (bcDNA) were then amplified and tagged with biotin via a two-step PCR (protocol 8). Presence of bcDNA both before and after amplification was checked by gel electrophoresis (protocol 9). Biotin-tagged strands were then isolated by binding to streptavidin beads and underwent pyrosequencing (protocol 10), which allows real time sequencing by synthesis. The pyrosequencing output is a percentage of DNA methylation at each CpG site, i.e. the percentage of methylated cytosines at the CpG site in all cells in the sample. An average DNA methylation value was then obtained from all CpG sites within each promoter region and compared across experimental groups and time points.

4.3.3.1 DNA extraction and clean-up

4.3.3.1.1 Protocol 1: simultaneous DNA/RNA extraction from brain with TRIzol

4.3.3.1.1.1 Precipitation and separation of RNA and DNA

- Transfer samples from -80°C storage to wet ice
- Transfer tissue to FastPrep matrix tubes with lysing beads (MPBio, Matrix D for animal tissues)
- Add 1 ml TRIzol (Invitrogen) per 50-100 mg sample
- Homogenise using FastPrep FP120 homogeniser (Thermo) (3 cycles at 5 m/sec for 15 sec)
- Incubate at room temperature (RT) for 5 min
- Add 0.2 ml chloroform per 1 ml Trizol and shake by inversion for 15 sec
- Incubate at RT for 2-3 min
- Centrifuge at <4000 rpm, 4°C for 15 min with hinge facing outward relative to the rotor
- Transfer the colourless upper aqueous phase (containing mRNA) to a fresh tube
- Store remaining sample (interphase containing DNA and organic pink phase containing proteins) at 4°C until DNA extraction

4.3.3.1.1.2 Washing and resuspending of RNA

See Chapter 3

4.3.3.1.1.3 Washing and resuspending of DNA

Refer to Invitrogen, TRIzol Reagent – Experimental Protocol for DNA Isolation

- Remove any remaining aqueous phase overlying the interphase (critical for DNA quality)
- Add 0.3 ml 100% ethanol per 1 ml Trizol and mix by gently inverting several times
- Incubate at RT for 3 min
- Transfer from matrix tubes to fresh 1.5 ml tubes
- Centrifuge at 2000 x g (4300 rpm) for 5 min to pellet the DNA
- Transfer the phenol-ethanol supernatant (containing proteins) to a fresh tube and store at -80°C
- Precipitation in 0.1 M sodium citrate in 10% ethanol (repeat this step at least twice, or three times for large pellets > 200 µg)
    - Resuspend the pellet in 1 ml of 0.1 M sodium citrate in 10% ethanol (pH 8.5) per 1 ml Trizol and mix by pipetting up and down

    - Incubate on a tube roller for 30 min (DNA can be stored in sodium citrate for at least 2 hours)
    - Centrifuge at 2000 x g (4300 rpm) for 5 min
    - Discard the supernatant
- Resuspend the pellet in 1.5 ml 75% ethanol per 1 ml Trizol
- Incubate on a tube roller for 10-20 min (DNA can be stored in 75% ethanol for several months at 4°C)
- Centrifuge at 2000 xg (4300 rpm) for 5 min
- Discard the supernatant
- Air dry the DNA pellet (can invert with lid open on blotting paper)
- Resuspend the pellet in 0.3 ml of 8 mM NaOH and mix by pipetting up and down
- Centrifuge at 12,000 xg (10,600 rpm) for 10 min to remove insoluble materials
- Transfer supernatant to a new tube
- Quantify and check purity of DNA on Nanodrop (Nanodrop 8000, Thermo Fisher Scientific) (average at least 2 measurements, NaOH as blanking buffer)
- Store at -20°C
- Proceed with DNeasy column clean-up (protocol 5)

4.3.3.1.2 Protocol 5: clean-up of brain DNA

- DNeasy Blood & Tissue kit column clean-up (Qiagen) (as per manufacturer instructions except for final elution volume)
    - Add 200 µl Buffer AL, mix by vortexing
    - Add 200 µl 100% ethanol, mix by vortexing
    - Transfer sample in a spin column (supplied)
    - Centrifuge at 6,000 xg (8,000 rpm) for 1 min
    - Discard flow-through and place column in a new collection tube (supplied)
    - Add 500 µl Buffer AW1
    - Centrifuge at 6,000 xg (8,000 rpm) for 1 min
    - Discard flow-through and place column in a new collection tube (supplied)
    - Add 500 µl Buffer AW2
    - Centrifuge at 20,000 xg (14,000 rpm) for 3 min
    - Discard flow-through very carefully to avoid ethanol carryover and place column in a new collection tube with lid cut (not supplied, prepare 2 sets)

- Elute in 100 µl Buffer AE in two steps (lower than 200 µl in single elution step as per manufacturer instructions)
    - Add 50 µl Buffer AE directly on the membrane
    - Incubate at RT for 2 min
    - Centrifuge at 6,000 xg (8,000 rpm) for 1 min and repeat step
- Check DNA purity on Nanodrop (Nanodrop 8000, Thermo Fisher Scientific) and measure DNA concentration on Qubit (Thermo Fisher Scientific) using the Qubit Broad Range dsDNA kit
- Proceed with bisulfite conversion (protocol 7)

4.3.3.1.3 Protocol 6: extraction and clean-up of blood DNA

- DNeasy Blood & Tissue kit column clean-up (Qiagen) (as per manufacturer instructions except for final elution volume)
    - Add 20 µl proteinase K to a 1.5 ml tube
    - Vortex defrosted anticoagulated blood briefly to mix plasma and cellular component
    - Add 50 µl blood
    - Adjust volume to 220 µl with phosphate-buffered saline (add 170 µl)
    - Add 200 µl Buffer AL, mix briefly by vortexing
    - Incubate at 56°C for 10 min
    - Continue with protocol 5 from step 2 (i.e. add 200 µl 100% ethanol)

4.3.3.2 Bisulfite conversion of genomic DNA from brain and blood

Bisulfite conversion requires a relatively precise input DNA concentration of 200-500 ng DNA. Precise measurements of DNA concentration required for downstream methylation analyses were obtained with the Qubit Fluorometer (Thermo Fisher Scientific) using the Qubit Broad Range dsDNA kit. DNA purity was checked with the Nanodrop (Nanodrop 8000, Thermo Fisher Scientific). Cleaned DNA from both brain and blood was bisulfite-converted (bcDNA) using the EZ DNA Methylation-Gold kit (Zymo Research). Bisulfite conversion chemically alters DNA and allows differentiation between methylated cytosines (protected) and unmethylated cytosines (converted to uracil). Presence of bcDNA was verified by agarose gel electrophoresis (protocol 8)

4.3.3.2.1 Protocol 7: bisulfite conversion of genomic DNA

- Measure DNA concentration on Qubit using the Qubit Broad Range dsDNA kit (Thermo Fisher Scientific)
- Prepare 500 ng DNA in 20 µl nuclease-free water (optimal DNA input required for EZ DNA Methylation-Gold kit, Zymo Research) and follow manufacturer instructions
    - Add 130 µl CT Conversion Reagent to 20 µl DNA, mix by pipetting up and down and spin down
    - Load samples on a thermal cycler (Bio-Rad) with the following steps:

Temperature	Time
98°C	10 min
64°C	2.5 hours
4°C	For optional storage up to 20 hours

- Check presence of bcDNA by gel electrophoresis (protocol 8)
- Amplify bcDNA immediately if possible, store remaining bcDNA at -20°C

4.3.3.2.2 Protocol 8: checking presence of bcDNA before and after PCR amplification by gel electrophoresis

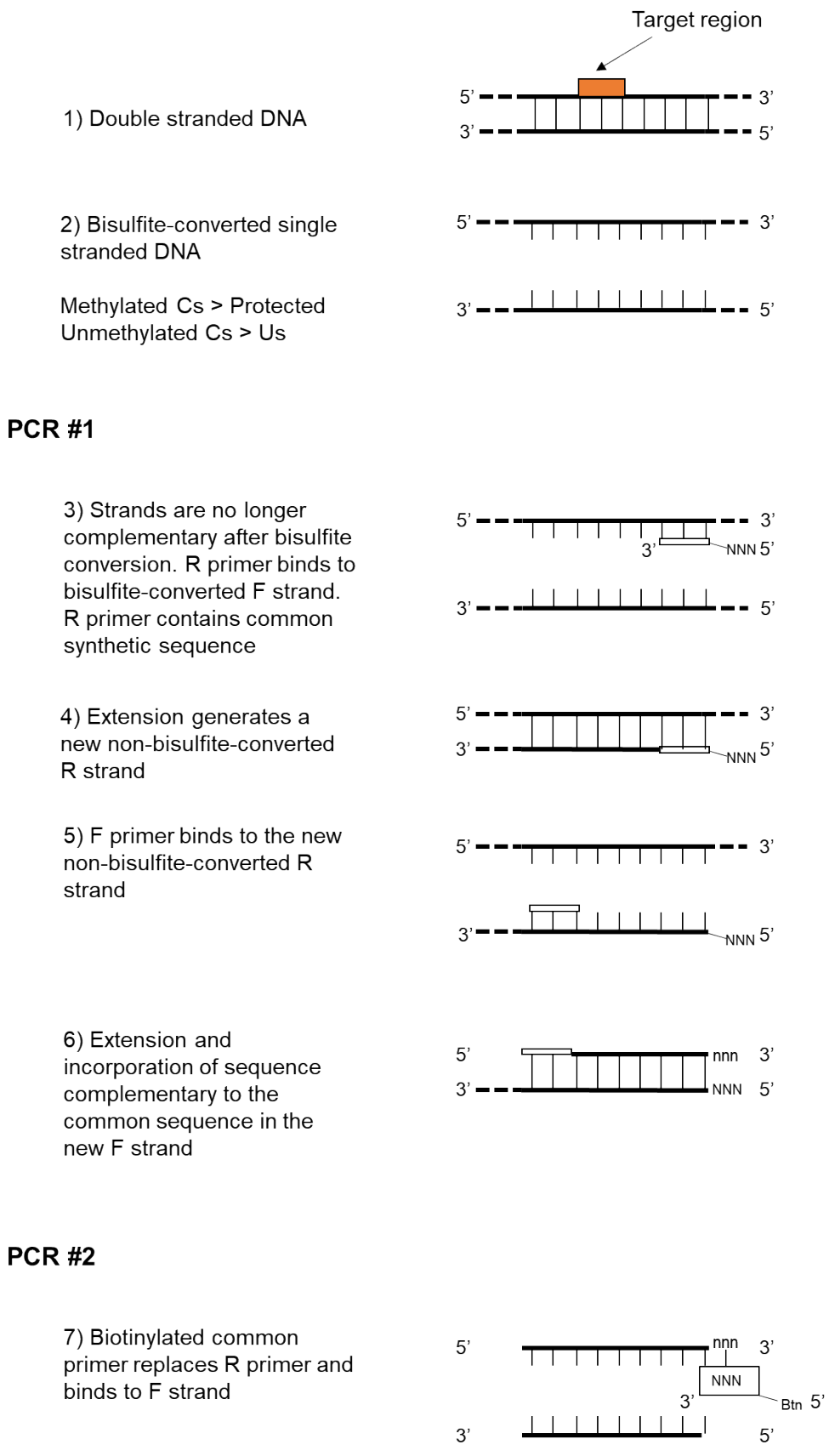
- Prepare a 1% agarose gel in 0.5X TE Buffer (e.g. 2 g in 200 ml TE Buffer) with either ethidium bromide or GelRed (Biotium) (5 µl/100 ml gel) and leave at RT to set until opaque
- Mix 2 µl samples with 0.5 µl loading dye (Bioline)
- Place the gel cast in the electrophoresis tank and submerge with 0.5X TE Buffer
- Load a DNA sample into each well and at least one well per row with a 100 bp DNA ladder (Bioline)
- Run at 120 V for at least 1 hour or until DNA ladder bands of interest are sufficiently separated to allow for size determination
- Visualise on a UV transilluminator
    - For bcDNA before amplification: chill gel on ice for a few minutes before visualisation (as per manufacturer instructions); presence of bcDNA is confirmed by the presence of a smear of fragments with a range of lengths (100-1,500 bp)
    - For bcDNA after amplification: strong and sharp DNA bands of expected size and minimal primer-dimer
- Proceed to PCR amplification of bcDNA (protocol 9)

4.3.3.3 PCR amplification and biotin-tagging of bcDNA from brain and blood

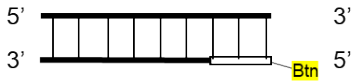
Typically, one of the primers used in PCR amplification (e.g. reverse primer) is tagged with biotin, which subsequently binds to streptavidin beads allowing isolation of the target DNA strands for pyrosequencing. Biotinylated primers are expensive. Moreover, the volume of source bcDNA is limited (10 µl as per bisulfite conversion protocol) and a single PCR may in turn offer a limited volume of amplified products, which may result in running out of source bcDNA if samples fail and need repeating. To avoid purchasing five biotin-tagged primers (i.e. one for each candidate promoter region in *Glt1*, one for *Tnf* and one for *Peg3*) and to produce larger volumes of amplified PCR products available for analysis, PCR amplification was divided in two steps.

In the first step, the reverse primer for each region was designed to include an established common recognition sequence upstream of the primer sequence (Dr Kira Rienecker, personal communication), which is incorporated during amplification (Figure 4.2) (1295). In the second PCR, a biotinylated version of the common primer is used instead of the reverse primer, therefore allowing biotin tagging of the strand complementary to the target strand, which is further amplified. Three technical replicates were run for each sample in the second PCR. The target strand is subsequently synthesised and sequenced in real time during pyrosequencing. Alignment with the reference sequence reveals which cytosines have been protected (unmethylated) and which have been converted to uracils (unmethylated).

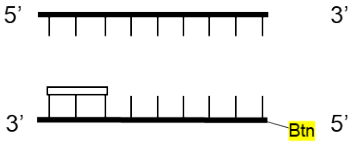
Figure 4.2. DNA methylation analysis: bisulfite conversion, two-step PCR, and pyrosequencing



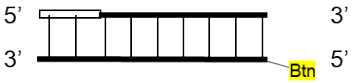
8) Extension of biotinylated R strand



9) Same F primer as PCR#1 binds to the R strand

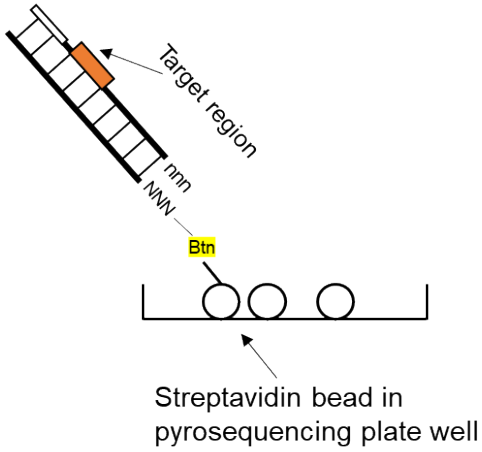


10) Extension of the F strand complementary to the biotinylated strand



Pyrosequencing

11) Biotinylated strands are selectively bound by streptavidin beads. Sequencing primer binds to the R biotinylated strand and extension generates a complementary strands including the target sequence



4.3.3.3.1 Primers

Primers for both PCR amplification and pyrosequencing were designed using the Pyromark Assay Design 2.0 software (Qiagen). Sequences of PCR primers, pyrosequencing primers and common primer are reported in Table 4.1. Whenever possible, primers were designed to avoid overlapping CpG sites, since this can lead to preferential amplification of methylated or unmethylated DNA. Whenever this was not possible due to the nature of the sequence, a mix of primers containing all four bases (A+G+C+T=N) at the CpG site was used (in red in Table 4.1), since this method has been previously shown to produce the least bias at similar melting temperatures (1296). The Pyromark Assay Design software outputs a quality score for each assay based on a range of *in silico* quality control analyses, with quality scores below 70 generally not recommended. Due to the nature of the *Glt1* promoter sequence, it was not possible to generate assays with a quality score above 70.

Studies of the *Peg3* differentially methylated region (DMR) (i.e. between maternal and paternal alleles) in the rat genome are scarce. In the mouse genome, the DMR has been studied more extensively, is approximately 4 kb long and spans the first exons of both *Peg3* and *Usp29*, another imprinted gene in the *Peg3* imprinted domain (1297). The DMR of mouse and rat were aligned using the Ensembl genome browser (Ensembl release 101, (1291), GRCm38.p6 mouse genome assembly, Rnor_06 rat genome assembly). Alignment revealed similar though not identical genomic structure. The gene is located on chromosome 7 in the mouse and chromosome 1 in the rat. The order of the genes in the *Peg3* domain is the same, with opposite orientation. However, the first exon of *Peg3* is over 200 kb from *Usp29* in the rat, making the boundaries of the target region in the DMR unclear. A published study previously identified a small discrete region in the mouse *Peg3* DMR including 5 CpG sites and assessed it via bisulfite conversion and pyrosequencing (1298). BLASTing this sequence against the rat genome revealed that this region is approximately 12 kb upstream of the TSS and is conserved in the rat. Primers were designed to target this region.

Table 4.1. DNA methylation primers

These include forward (F) and reverse (R) PCR primers, and pyrosequencing (S) primer. Whenever it was not possible to avoid a CpG site, degenerate primers containing all four bases (N, in red) at the site were used. The common primer sequence is shown in blue. The quality score was automatically assigned by the Pyromark Assay Design 2.0 software

Primer	Sequence	PCR#2 Product Size	Score
common_biotin	Btn - CGCCAGGGTTTTCCCAGTCACGAC	24	
glt1_dist_F	GGTTGAAGAGAATATAAAGTTGAT	399 + 24 = 423	
glt1_dist_R	CGCCAGGGTTTTCCCAGTCACGACACATCTT CNCTTCTTTACATCCACTACA		68
glt1_dist_S	AAATAGGAGAGGGTG		
glt1_prox_F	GTTATTATTATGTAAAGTTGGGTATGAGAT	154 + 24 = 178	
glt1_prox_R	CGCCAGGGTTTTCCCAGTCACGACCCACTC CCCAAACCAAATACTAAAA		66
glt1_prox_S	AGTTTATTTGTTTTTGTATATTTT		
glt1_isl_F	GGTGATGTTAGTTTTGGATAAAAATAGAGA	272 + 24 = 296	
glt1_isl_R	CGCCAGGGTTTTCCCAGTCACGACCCTAAC CNCCACCTATATCTATACTTC		63
glt1_isl_S	GGGGTTAAATTTTGTAATTTTT		
tnf_F (not used)	TTGGAGAAGAAATTGAGAGAGGTGTAG	204 + 24 = 228	
tnf_R (not used)	CGCCAGGGTTTTCCCAGTCACGACACTATTC TCCCTCCTAACTAATCC		82
tnf_S (not used)	GGTTTTTTTTATTAAGGAAGTTTT		
peg3_F	TTGTTGTAGANGTTGGGGAGTTAAGA	125+24=149	
peg3_R	CGCCAGGGTTTTCCCAGTCACGACACAATC TAATACACCCACACTAA		82
peg3_S	TGGGGAGTTAAGAGT		

4.3.3.3.2 Protocol 9: two-step PCR amplification

All PCR assays were individually optimised prior to the experiment, and an annealing temperature of 57°C was found to be appropriate for all assays. The steps for the two PCRs are identical except for:

1) The template was bcDNA in PCR#1, and a 1:6 dilution of PCR#1 products in PCR#2
2) The gene/region-specific reverse primer was replaced with the biotinylated common primer in PCR#2
3) Volumes were doubled in PCR#2
4) Three technical replicates per sample were run in PCR#2

- PCR#1
    - Prepare master mix

PCR Reagents	Volume for one reaction	Working Concentration
Water	6.175 µl	
Buffer	1 µl	10X
dNTPs	0.8 µl	2 mM
F primer	0.3 µl	10 µM
R primer	0.3 µl	10 µM
HotStart Taq Polymerase	0.125 µl	5 U/µl

    - Add 1 µl bcDNA sample or no-template control (NTC) to 9 µl master mix
    - Load on a thermal cycler (BioRad) with the following steps:

PCR Cycle Step	Temperature	Time	No. Cycles
Taq Heat Activation	95°C	15 min	
Denaturation	95°C	30 sec	
Annealing	57°C	60 sec	X 25
Extension	72°C	40 sec	
Final Extension	72°C	10 min	
Hold	4°C	Indefinitely	

    - Check amplification by gel electrophoresis (protocol 8)

- PCR#2
    - Prepare master mix

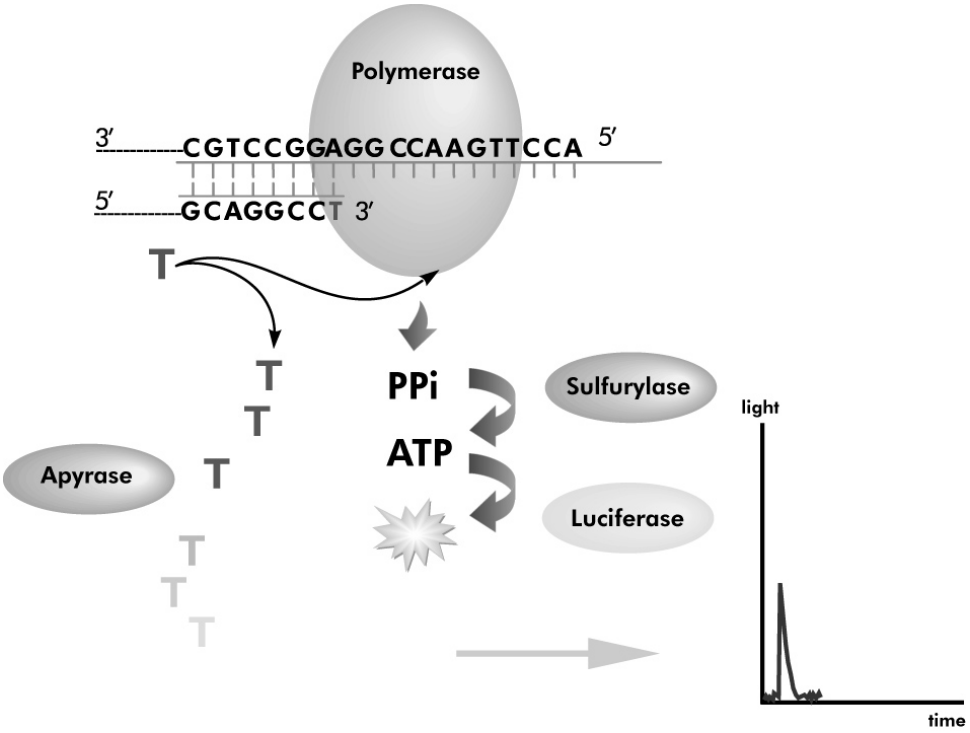
PCR Reagents	Volume for one reaction	Working Concentration
Water	12.35 µl	
Buffer	3.6 µl	10X
dNTPs	1.6 µl	2 mM
F primer (same as PCR#1)	0.6 µl	10 µM
R primer (biotinylated common primer)	0.9 µl	10 µM
HotStart Taq Polymerase	0.25 µl	5 U/µl

    - Add 1 µl of 1:6 dilution of PCR#1 product or no-template control (NTC) to 9 µl master mix
    - Load on a thermal cycler (BioRad) with the same steps as PCR#1
    - Check amplification by gel electrophoresis (protocol 8)

4.3.3.4 Pyrosequencing

Samples (PCR#2 products) were mixed with streptavidin beads to allow selective binding of the biotinylated DNA strands. These were subsequently transferred to a pyrosequencing plate containing the sequencing primer. Pyrosequencing was carried out with the Pyromark Q96 system (Qiagen). During the automated pyrosequencing process, the sample is flushed with a pre-specified sequence of deoxyribonucleoside triphosphates (dNTPs); if the dNTP is complementary to the template strand, it is added by DNA polymerase (Figure 4.3). Every time a dNTP is incorporated, pyrophosphate (PPi) is released in a quantity that is equimolar to the number of incorporated nucleotides, i.e. twice as much pyrophosphate if the sequence includes two of the same dNTPs in a row. The enzyme sulfurylase converts pyrophosphates to ATP, while the enzyme apyrase degrades any unincorporated nucleotides. The enzyme luciferase converts ATP to a light signal, which is recorded by a camera in the Pyromark Q96 and reported as a peak on a pyrogram. Peaks of different colours and lengths depending on the type and number of consecutive dNTPs allow to infer the target sequence.

Figure 4.3. Pyrosequencing cascade as illustrated on the Pyromark Gold Q96 Reagents handbook (1299)



Prior to the experiment, all pyrosequencing assays were validated by running spare samples with various controls to rule out unwanted background signals, i.e. PCR reagents without template (duplexes between PCR primers), all pyrosequencing reagents minus the sequencing

primer (duplexes between PCR products), all pyrosequencing reagents minus the template and sequencing primer (duplexes or hairpins in the sequencing primer), all pyrosequencing reagents minus the template (duplexes between the sequencing and biotinylated reverse primer).

4.3.3.4.1 Protocol 10: Pyrosequencing

- Create an assay file for each target sequence by inputting information from the Pyromark Assay Design 2.0 software into the Pyromark Q96 software. This file allows the Pyromark Q96 software to calculate the appropriate volumes of enzyme, substrate, and nucleotides to load on the Pyromark Q96 cartridge (code 0016, Qiagen) depending on the number of samples on the 96-well pyrosequencing plate (Qiagen)
- Prepare Pyromark Gold reagents as per manufacturer instructions, i.e. add 620 ul water to both enzyme and substrate, swirl gently and leave at RT for 5-10 min; Pyromark Gold dNTPs are ready for use
- Prepare the streptavidin beads master mix (keep vortexing/inverting regularly to prevent the beads from settling at the bottom of the tube)

Streptavidin Beads Master Mix Reagents	Volume for one reaction
Streptavidin Beads	1.5 µl
Pyromark Binding Buffer	40 µl
Water	18.5 µl

- Add 60 µl streptavidin beads master mix and 2 µl water to each sample (~18 µl after gel electrophoresis) in the 96-well PCR plate (final volume 80 µl)
- Agitate PCR plate on a plate shaker at 1400 rpm for 5-10 min to ensure binding of biotinylated primers to streptavidin beads
- Prepare 0.4 uM sequencing primer master mix

0.4 uM Sequencing Primer Master Mix Reagents	Volume for one reaction
Pyromark Annealing Buffer	38.4 µl
Sequencing Primer (10 uM Stock)	1.6 µl

- Add 40 µl to each well of a pyrosequencing plate (same layout as PCR#2 plate with three technical replicates)
- Set up Pyromark Vacuum Station according to manufacturer instructions, by placing the PCR plate, pyrosequencing plate and reservoirs in the specified orientation, and filling up the reservoirs with 70% ethanol, Pyromark Denaturation Buffer, Pyromark Wash Buffer, and nuclease-free water

- Use the Pyromark vacuum tool to transfer the beads from the PCR plate to the pyrosequencing plate with the sequencing primer
    - With vacuum tool in parked position, switch ON vacuum machine and vacuum switch and flush all water in the reservoir
    - With vacuum ON, insert tool carefully into PCR plate wells to draw the beads to the filter probes of the vacuum tool, holding for 15 seconds and minimising any contact between the probes and the walls of the well
    - With vacuum ON, transfer the vacuum tool in the various stations in sequence:
        - Ethanol for 5 sec
        - Denaturation Buffer for 5 sec
        - Wash Buffer for 10 sec
        - Turn the tool facing up past 90°C angle and hold for 5 sec to dry
        - Align and hover over the pyrosequencing plate, turn vacuum machine off, remove hose, lower vacuum tool into the plate and agitate/tap for 15 seconds
- Place the pyrosequencing plate on a pyrosequencing thermoplate (Qiagen), cover loosely (e.g. with a PCR plate seal) to avoid evaporation and place on a heat block at 80°C for 2 minutes to allow annealing of the sequencing primer to the single strands of DNA; leave to cool at RT
- Load required volumes of Pyromark Gold reagents (enzyme, substrate, and dNTPs) on the Pyromark cartridge, load plate on the Pyromark Q96 and run pyrosequencing
- Repeat samples identified by the software as low quality and colour-coded in red; manually inspect samples colour-coded in yellow and repeat if appropriate; include samples colour-coded in blue in the analysis
- For each CpG site, the Pyromark Q96 output is the percentage of methylated cytosines in the population of cells in the sample. For example, a CpG site that is 50% methylated followed by a non-CpG T will give a peak of 50% for C and 150% for T

4.3.4 Statistical analysis

The exposures in this study were experimental group (naïve, sham, hypoxia-ischaemia) and time (6h, 12h, 24h), while the outcome was the percentage DNA methylation at each of the promoter regions (three for *Glt1* and one for *Peg3*) investigated in both left cortex and blood. The percentage methylation at each region was calculated by averaging methylation at multiple CpG sites included in each region. This is based on previous evidence that in

promoters there is strong correlation between neighbouring CpG within 1-2 kb regions in the same cell types, and measurements of DNA methylation tend to not be independent (1300-1304). Differentially methylated regions composed of correlated CpG sites exist in the context of different tissues, developmental stages, disease and ageing (1305). Reducing data to regions can therefore increase power and reduce noise (1306). Single CpG sites were still examined in detail both graphically with scatterplots of raw DNA methylation data and at secondary analyses stage in case of significant findings at the region level. The number of CpG sites in each promoter region varies throughout the study, since it depends on the characteristics of each DNA sequence (CpG density and distance between sites) and on the limit in sequence length that can be studied by pyrosequencing (up to 60-80 bp).
The percentage methylation at each CpG site is derived from all cells in the sample. While on a single DNA sequence on a single chromosome, methylation at a CpG site is binary (either 0% or 100% methylated), each cell has two chromosomes, so that the same CpG site in a single cell can be 0%, 50% or 100% methylated. Each sample in this study is a section of bulk brain tissue or a specified volume of blood, containing DNA sequences from many thousands of cells, including different cell types with variable methylation status. Therefore, the percentage methylation at each CpG site in a sample will be a snapshot of methylation in all the cells in the sample, i.e. the meta-epigenome. As bisulfite conversion is not error-free, slightly looser thresholds were set arbitrarily to indicate methylation states: 0-10% range was considered as unmethylated, a 90-100% range was considered as fully methylated, whereas a 10-90% range was considered as indicative of partial methylation. Thus, partial methylation may be due to methylation differences between the two chromosomes of a single cell, between cells of the same type, and between different types of cells.

Firstly, the percentage methylation at each CpG site in each candidate region was plotted to graphically assess whether averaging to obtain a single methylation value per region was appropriate. The methylation across all CpG sites within each region was then averaged to obtain a single methylation value per region for each rat, which was used in the statistical analyses. A two-way 3 x 3 factorial ANOVA was run separately on 42 left cortices and related blood samples to compare the main effects and interaction of experimental group (naïve, sham, hypoxia-ischaemia) and time (6h, 12h, 24h) on percentage DNA methylation at each candidate region. The Scheffe's test was used for *post hoc* analyses to identify which pairs of means were significantly different, since, although it affords less power than the Tukey's and Fisher's tests, it allows to compare all possible pairs of three means. Effect sizes

were expressed in terms of population (ω^2, omega squared) rather than sample (η^2, eta squared) effect sizes, as, though always smaller, they have been found to be carry less sampling error bias in unbalanced designs with multiple groups (1213).

Secondary analyses of suggestive value included:

- A 2 x 3 ANOVA collapsing naïve (n=3 per timepoint) and sham (n=3 per timepoint) into a single control group (n=6 per time point) to increase power, as long as no differences were found between naïve and sham. These analyses were carried out in the cortex and followed up in blood depending on the findings in the cortex
- A 3 x 3 ANOVA repeated for each individual CpG site in the region, whenever primary analyses yielded significant findings; this allowed to ascertain whether differences were driven by specific CpG sites within the region or the region as a whole

Uncorrected p-values are reported. A Benjamini Hochberg False Discovery Rate was applied for significant findings with a false discovery rate of 5% (1214).

All statistical analyses and plots were carried out using Stata 14 (Stata Corp, TX, USA).

4.4 Results

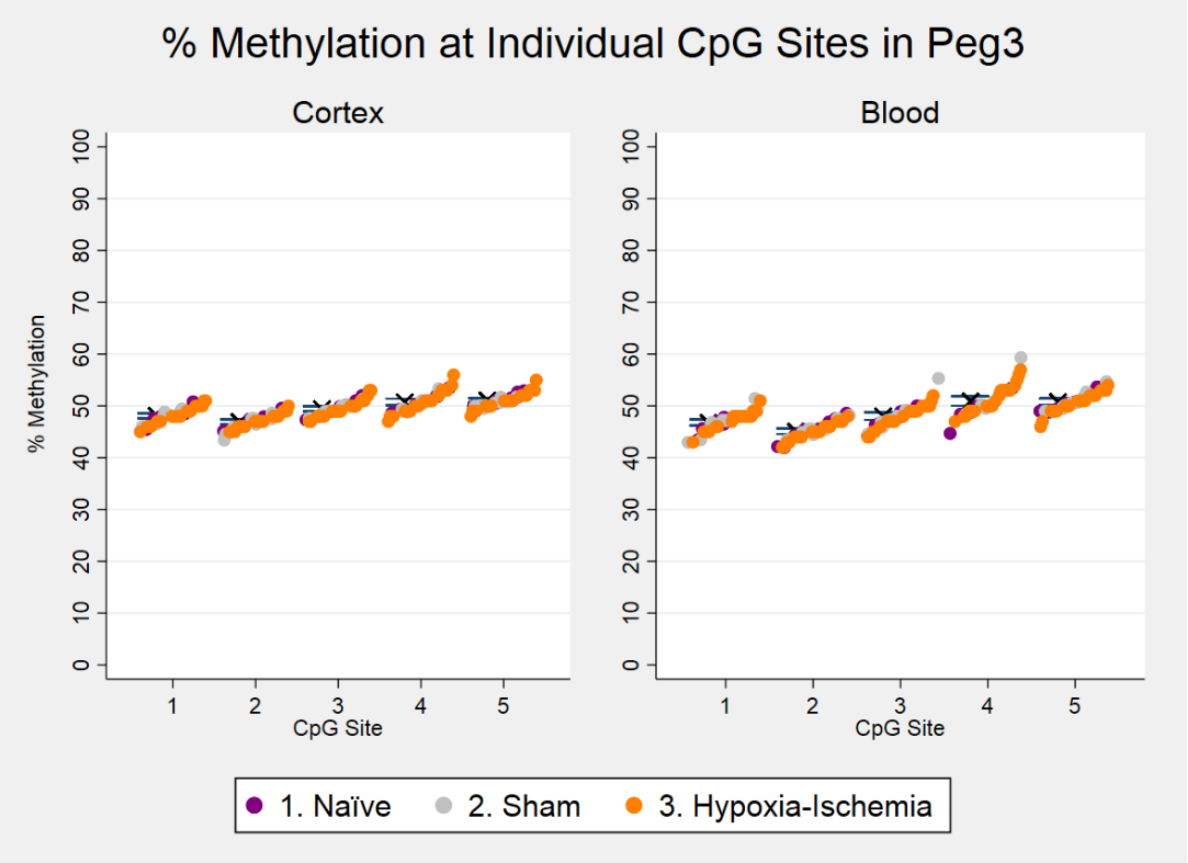
4.4.1 Peg3

Methylation at the 5 CpG sites in the *Peg3* promoter was between 45% and 51%, with very little variability and SD no larger than 3% (Table 4.2, Figure 4.4). When methylation was averaged across all 5 CpG sites, the mean percentage methylation across the *Peg3* promoter was ~50% in both cortex and blood.

Table 4.2. *Peg3* – Percentage DNA methylation at individual CpG sites

***Peg3*: CpG Site**	**n**	**Cortex**		**Blood**	
		Mean % DNAm	**SD**	**Mean % DNAm**	**SD**
1	42	48.13	1.52	46.78	1.90
2	42	46.95	1.50	45.10	1.72
3	42	49.43	1.57	48.03	2.29
4	42	50.75	2.03	50.95	2.91
5	42	51.10	1.37	50.83	1.85

Figure 4.4. *Peg3* – Percentage DNA methylation at individual CpG sites



4.4.2 Glutamate transporter

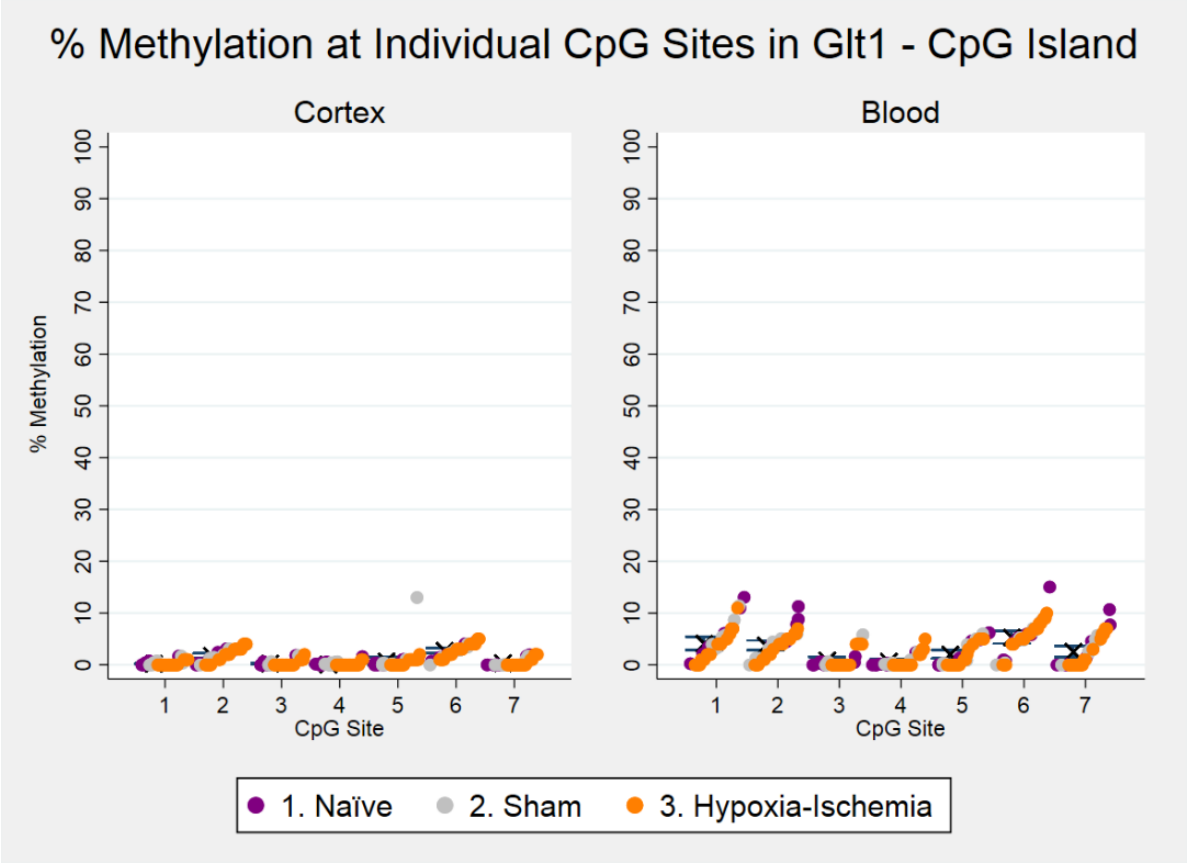
4.4.2.1 CpG island

The CpG island was largely unmethylated across all 7 CpG sites, with mean methylation for each CpG site below 3% in the cortex and below 6% in blood. Variability was higher in blood but still in the low range, with standard deviations no larger than 4% (Table 4.3, Figure 4.5). Of note, CpG 7 coincides with the -181 SNP in humans, and is a CpG site only in rats.

Table 4.3. *Glt1* (CpG island) – Percentage DNA methylation at individual CpG sites

***Glt1* (CpG island): CpG Site**	**n**	**Cortex**		**Blood**	
		Mean % DNAm	**SD**	**Mean % DNAm**	**SD**
1	42	0.22	0.42	4.15	3.67
2	42	1.78	1.38	3.79	2.67
3	42	0.25	0.50	0.94	1.71
4	42	0.08	0.28	0.67	1.24
5	42	0.72	2.17	2.06	2.32
6	42	2.75	1.38	5.33	3.41
7 (EAAT2 -181)	42	0.36	0.68	2.58	2.97

Figure 4.5. *Glt1* (CpG island) – Percentage DNA methylation at individual CpG sites



When methylation was averaged across all 7 CpG sites (i.e. one methylation value across the entire region for each rat), the *Glt1* CpG island region resulted unmethylated (< 5%) in both cortex and blood across all groups and time points (Table 4.4, Figure 4.6). There were no significant differences in percentage methylation at the CpG island between groups and time points in primary or secondary analyses (Table 4.5, Table 4.6).

Table 4.4. *Glt1* (CpG island): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; DNAm: DNA methylation; SD: standard deviation

Group	Time	n	*Glt1* – CpG island			
			Cortex		Blood	
			Mean % DNAm	SD	Mean % DNAm	SD
N	6	3	0.64	0.51	2.21	0.91
	12	3	0.57	0.49	4.28	0.66
	24	3	1.00	0.49	3.21	0.10
S	6	3	1.21	1.52	3.29	0.40
	12	3	0.81	0.58	3.48	0.70
	24	3	0.95	0.30	1.95	1.57
HI	6	8	0.73	0.38	2.29	0.88
	12	8	0.83	0.51	2.45	1.26
	24	8	1.19	0.37	3.04	1.32

Figure 4.6. *Glt1* (CpG island) DNA methylation: plots

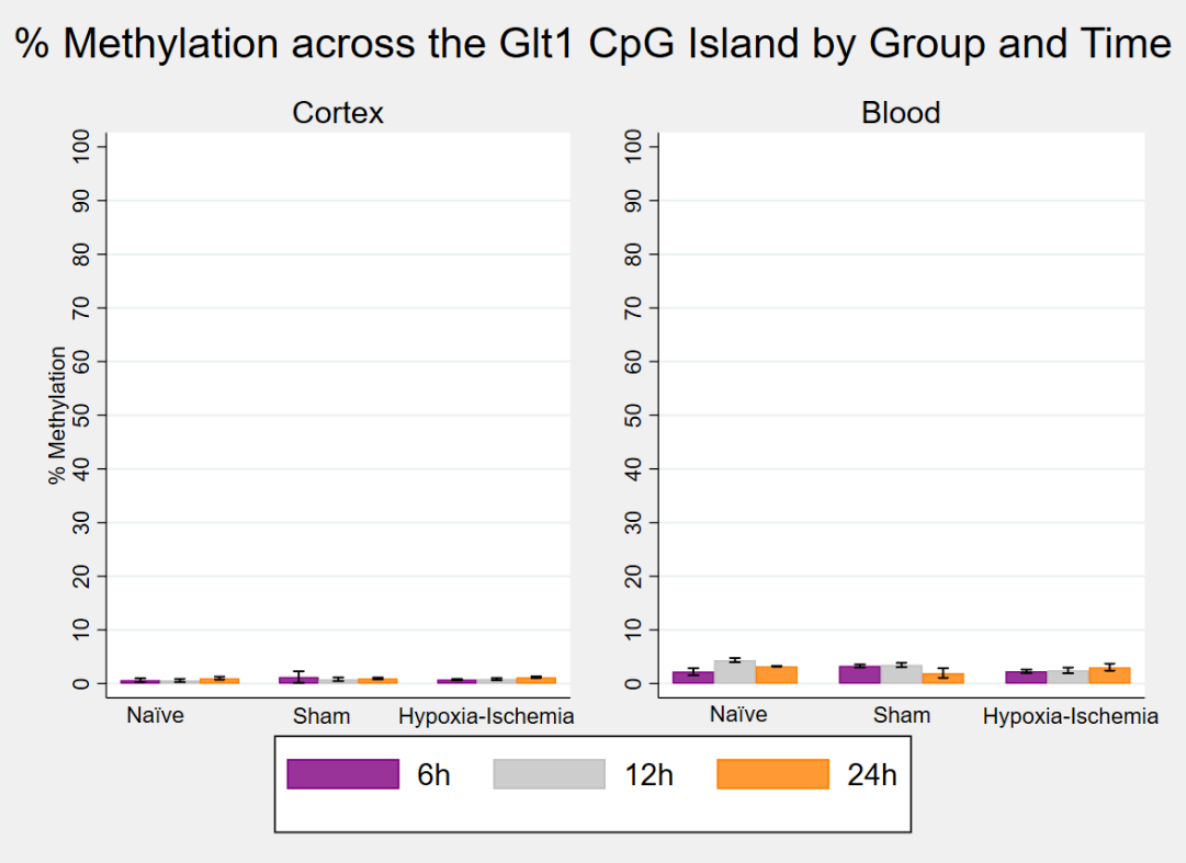


Table 4.5. *Glt1* (CpG island): primary analyses

Gene	Tissue	Variable	Levene's p	df	MS	F	p	Effect Size (omega^2)
***Glt1* (CpG island)**	***Cortex***	*Group (N vs S vs HI)*	0.001	2	0.14	0.50	0.611	0 (-0.03)
		Time		2	0.26	0.96	0.397	0 (-0.003)
		*Group*Time*		4	0.12	0.46	0.767	0 (-0.07)
	Blood	*Group (N vs S vs HI)*	0.47	2	1.15	1.05	0.367	0.003
		Time		2	1.97	1.80	0.187	0.06
		*Group*Time*		4	2.14	1.96	0.133	0.12

Table 4.6. *Glt1* (CpG island): secondary analyses

Gene	Tissue	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
***Glt1* (CpG island)**	***Cortex***	*N vs S*	*Group*	0.01	1	0.25	0.57	0.468	0 (-0.04)
			Time		2	0.14	0.31	0.740	0 (-0.12)
			*Group*Time*		2	0.12	0.26	0.773	0 (-0.13)
		N/S vs HI	*Group*	0.09	1	0.02	0.10	0.759	0 (-0.03)
			Time		2	0.34	1.31	0.284	0.02
			*Group*Time*		2	0.13	0.51	0.603	0 (-0.03)

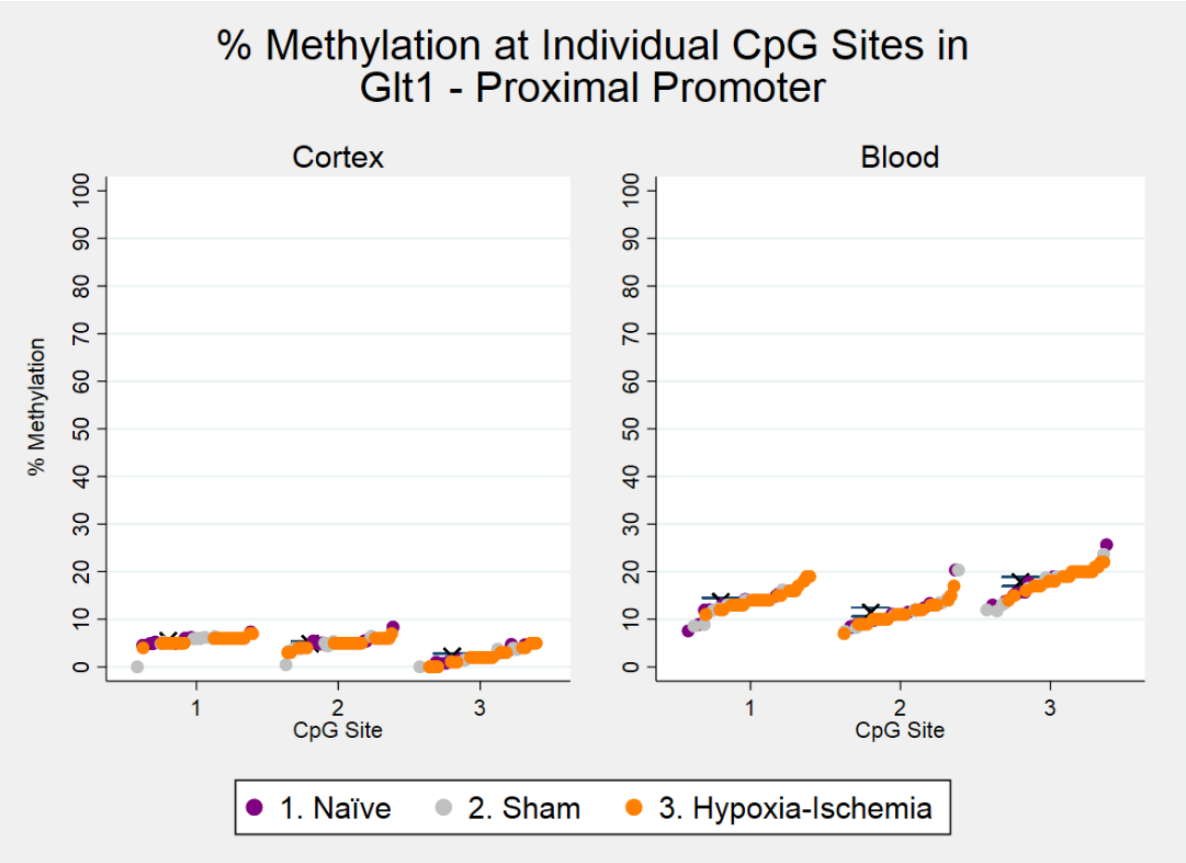
4.4.2.2 Proximal promoter

The proximal promoter was largely unmethylated across all 3 CpG sites in the cortex, with mean methylation for each CpG site below 6% and standard deviations no larger than 2%. Methylation in blood was higher but still in the low range, with mean percentage methylation for each CpG site below 18% and standard deviations no larger than 4% (Table 4.7**,** Figure 4.7). Of note, CpG 3 coincides with a CpG site (cg21163960) that is differentially methylated in the saliva of very preterm newborns vs healthy term newborns in an EWAS (1292).

Table 4.7. *Glt1* (proximal promoter) – Percentage DNA methylation at individual CpG sites

***Glt1* (proximal promoter): CpG Site**	**n**	**Cortex**		**Blood**	
		Mean % DNAm	**SD**	**Mean % DNAm**	**SD**
1	42	5.52	1.09	13.74	2.57
2	42	4.95	1.27	11.52	2.92
3 (Differentially Methylated in (1292))	42	2.29	1.53	17.90	3.11

Figure 4.7. *Glt1* (proximal promoter) – Percentage DNA methylation at individual CpG sites



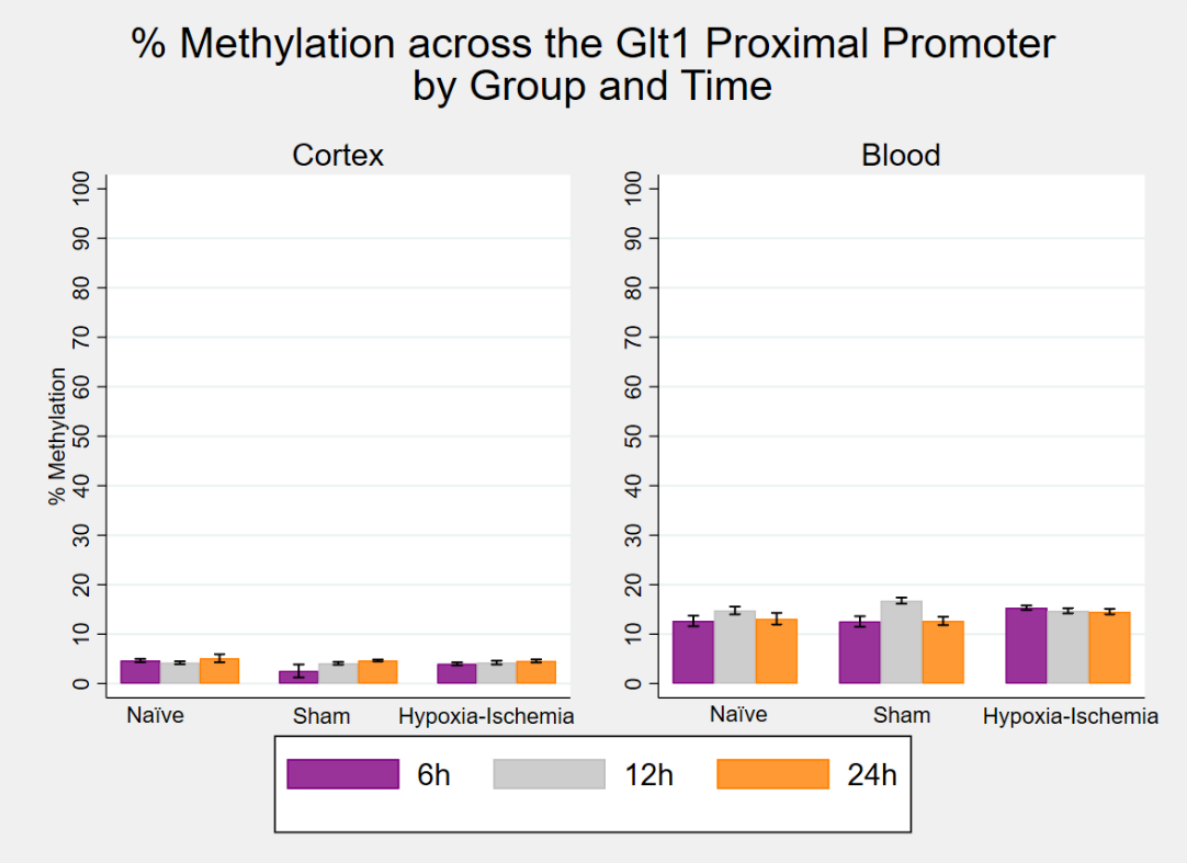
When methylation was averaged across all 3 CpG sites, the *Glt1* proximal promoter resulted unmethylated in the cortex (<5%) and hypomethylated in blood (<20%) across all groups and time points (Table 4.8, Figure 4.8).

Table 4.8. *Glt1* (proximal promoter): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; DNAm: DNA methylation; SD: standard deviation

Group	Time	n	*Glt1* – proximal promoter			
			Cortex		Blood	
			Mean % DNAm	SD	Mean % DNAm	SD
	6	3	4.67	0.58	12.67	1.86
N	**12**	3	4.22	0.51	14.78	1.35
	24	3	5.11	1.39	13.11	2.04
	6	3	2.56	2.27	12.56	1.84
S	**12**	3	4.11	0.51	16.78	1.07
	24	3	4.67	0.33	12.67	1.45
	6	8	4.00	0.87	15.33	1.30
HI	**12**	8	4.25	1.09	14.71	1.43
	24	8	4.58	0.87	14.54	1.56

Figure 4.8. *Glt1* (proximal promoter) DNA methylation: plots



In the cortex, there was very weak evidence of a time effect that was independent of group (p=0.065), with *post hoc* analysis suggesting the difference may be between 6h and 24h (p=0.098) (Table 4.9). However, the difference in methylation was less than 1%. Therefore, similar to the CpG island, the proximal promoter was largely unmethylated across groups and time points.

In blood but not brain, there was a significant interaction between group and time on percentage methylation (p=0.018). Simple main effect analysis suggested that there were significant differences by group at 6h (p=0.004), with *post hoc* analysis suggesting that HI rats had higher methylation than both naïve and sham rats at 6h. The difference in means was less than 3% in both cases, with methylation therefore remaining in the low range. Any evidence of differences between groups disappeared at 12h (p=0.11) and 24h (p=0.12).

Table 4.9. *Glt1* (proximal promoter): primary analyses

Gene	Tissue	Variable	Levene's p	df	MS	F	p	Effect Size	*Post hoc* Scheffe	*Post hoc Mean Difference (95% CI)*
***Glt1* (proximal promoter)**	***Cortex***	*Group (N vs S vs HI)*	0.05	2	1.79	1.69	0.201	0.04		
		Time		2	3.13	2.94	0.067	0.10	6h may be ≠ 24h (p=0.098)	24h – 6h = 0.89% (-0.001-1.76%)
		*Group*Time*		4	1.07	1.01	0.419	0.001		
	Blood	*Group (N vs S vs HI)*	0.98	2	6.77	2.97	0.065	0.10		
		Time		2	14.31	6.27	0.005	0.23		
		*Group*Time*		4	7.87	3.45	**0.018** Simple Main Effects: Group*6h (p=0.004)	0.21	At 6h, N (p=0.073) and S (p=0.061) ≠ HI	At 6h, HI – N = 2.67% (-1.66-6.99%) At 6h, HI – S = 2.78% (-1.55-7.10)

In the secondary analyses collapsing naïve and sham groups, there was no significant evidence of differences in DNA methylation at the *Glt1* proximal promoter in the cortex (Table 4.10).

Table 4.10. *Glt1* (proximal promoter): secondary analyses

Gene	Tissue	Design of secondary analysis	Variable	Levene's p	df	MS	F	p	Effect size
***Glt1* (proximal promoter)**	***Cortex***	*N vs S*	*Group*	0.01	1	3.56	2.65	0.129	0.11
			Time		2	2.46	1.84	0.201	0.10
			*Group*Time*		2	1.72	1.29	0.312	0.04
		N/S vs HI	*Group*	0.29	1	0.03	0.03	0.870	0 (-0.03)
			Time		2	2.99	2.56	0.092	0.07
			*Group*Time*		2	0.42	0.36	0.703	

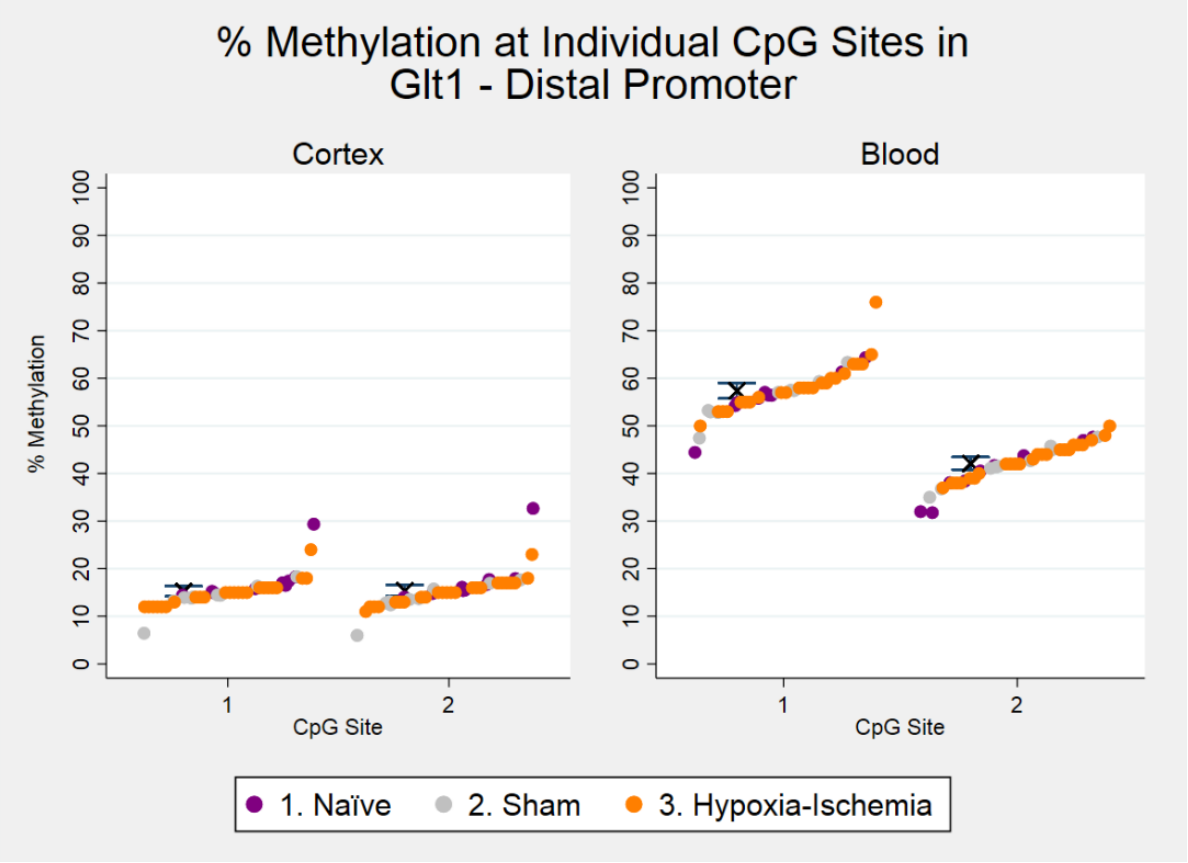
4.4.2.3 Distal shore

The distal promoter of *Glt1* was hypomethylated at both CpG sites in the cortex, with mean percentage methylation for each CpG site around 15% and standard deviations no larger than 4%. Methylation in blood was higher and in the mid-range, with a mean of 57% for one CpG site and 42% for the other CpG site, and standard deviations no larger than 6% (Table 4.11, Figure 4.9).

Table 4.11. *Glt1* (distal shore) – Percentage DNA methylation at individual CpG sites

***Glt1* (distal promoter): CpG Site**	**n**	**Cortex**		**Blood**	
		Mean % DNAm	**SD**	**Mean % DNAm**	**SD**
1	42	15.26	3.44	57.40	5.23
2	42	15.43	3.82	42.14	4.21

Figure 4.9. *Glt1* (distal shore) – Percentage DNA methylation at individual CpG sites



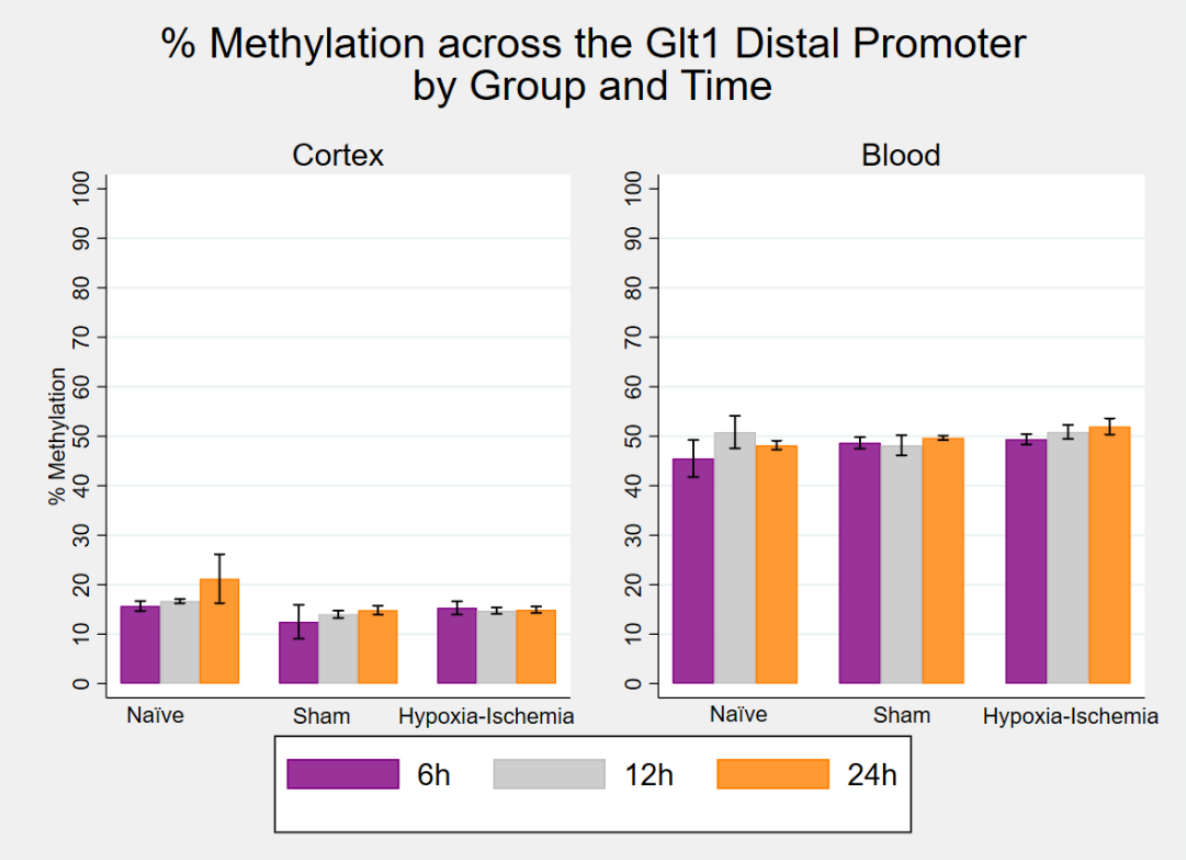
When methylation was averaged across both CpG sites, the *Glt1* distal promoter resulted hypomethylated in the cortex (<20%) and around 50% methylated in blood across all groups and time points (Table 4.12, Figure 4.10).

Table 4.12. *Glt1* (distal shore): descriptive statistics

N: naïve; S: sham; HI: hypoxia-ischaemia; DNAm: DNA methylation; SD: standard deviation

Group	Time	n	*Glt1* – distal shore			
			Cortex		Blood	
			Mean % DNAm	SD	Mean % DNAm	SD
N	6	3	15.67	1.76	45.50	6.50
	12	3	16.67	0.76	50.83	5.69
	24	3	21.17	8.55	48.17	1.53
S	6	3	12.50	5.89	48.67	2.02
	12	3	14.00	1.32	48.17	3.55
	24	3	14.83	1.53	49.67	0.76
HI	6	8	15.31	3.75	49.38	2.97
	12	8	14.75	1.81	50.88	3.99
	24	8	14.94	1.84	51.94	4.59

Figure 4.10. *Glt1* (distal shore) DNA methylation: plots



In the cortex, there was significant evidence of a group effect (p=0.041, effect size: 12%), with *post hoc* analysis suggesting the difference was between naïve and sham rats only

(p=0.047) (Table 4.13). Specifically, sham rats had 4% lower methylation at the distal promoter compared to naïve rats. No group or time effects were detected in blood.

Table 4.13. *Glt1* (distal shore): primary analyses

Gene	Tissue	Variable	Levene's p	df	MS	F	p	Effect Size	*Post hoc* Scheffe's p	*Post hoc Mean Difference (95% CI)*
***Glt1* (distal shore)**	***Cortex***	*Group (N vs S vs HI)*	0.001	2	40.34	3.54	**0.041**	0.12	S ≠ N (p=0.047)	*S – N* = -4.06% (-8.07- -0.04)
		Time		2	18.92	1.66	0.206	0.04		
		*Group*Time*		4	10.92	0.96	0.444	0 (-0.04)		
	Blood	*Group (N vs S vs HI)*		2	26.56	1.72	0.194	0.04		
		Time		2	16.62	1.08	0.352	0.004		
		*Group*Time*		4	7.94	0.51	0.725	0 (-0.05)		

In the secondary analyses, the two individual CpG sites were analysed separately, and it was found that the level of evidence and the effect size were similar for both CpG sites (Table 4.14). No group or time effects were detected in blood.

Table 4.14. *Glt1* (distal shore): secondary analyses

Gene	Tissue	CpG Site	Variable	Levene's p	df	MS	F	p	Effect size	*Post hoc* Scheffe's p	*Post hoc Mean Difference (95% CI)*
***Glt1* (distal shore)**	***Cortex***	*1*	*Group (N vs S vs HI)*	0.004	2	36.30	3.37	**0.047**	0.12	S ≠ N (p=0.059)	*S – N* = -3.78% (-7.67-0.12)
			Time		2	11.06	1.03	0.370	0.001		
			*Group*Time*		4	12.53	1.16	0.345	0.02		
		2	*Group (N vs S vs HI)*	0.001	2	44.83	3.41	**0.045**	0.12	S ≠ N (p=0.050)	*S – N* = -4.33% (-8.67- -0.001)
			Time		2	28.91	2.20	0.127	0.06		
			*Group*Time*		4	9.60	0.73	0.578	0 (-0.03)		

4.4.2.4 Overall results

None of the group effects survived Benjamini Hochberg correction (FDR 5%) (Table 4.15).

Table 4.15. DNA methylation: findings surviving Benjamini-Hochberg False Discovery Rate (5%) correction (none)

Glt1 Promoter Region	Tissue	p-value for Group Effect (Primary Analyses)	Rank	Benjamini-Hochberg critical value
Distal	Cortex	0.041	1	0.01
Proximal	Blood	0.065	2	0.02
Distal	Blood	0.194	3	0.04
Proximal	Cortex	0.201	4	0.05
CpG Island	Blood	0.367	5	0.06
CpG Island	Cortex	0.611	6	0.07

4.5 Discussion

This exploratory study set out to assess whether hypoxia-ischaemia alters DNA methylation at the promoter of *Glt1* and *Tnfα*, potentially mediating latent or observed gene expression changes in the cortex. Correlation with DNA methylation in blood was also assessed as a potential early biomarker of injury. Only *Glt1* was assessed due to COVID-19-related disruptions. Overall, DNA methylation was not significantly altered in any of the three *Glt1* promoter regions assessed. Small methylation differences (<5%) of dubious biological relevance included: 1) proximal promoter in the cortex: higher methylation at 24h vs 6h; 2) proximal promoter in blood (but not cortex): higher methylation in HI rats at 6h 3) distal promoter in the cortex: lower methylation in sham vs naïve.

4.5.1 Peg3 data supports accuracy and precision of DNA methylation methodology

Bisulfite pyrosequencing is currently considered the gold standard method for candidate gene DNA methylation studies since it allows quantification at the individual cytosine level with relatively little bias (1307, 1308). With the progress in next-generation sequencing technologies this approach can be extended to the entire genome. However, it is a challenging methodology for multiple reasons. Firstly, bisulfite conversion chemically alters DNA to convert unmethylated cytosines to uracils, which are subsequently read as thymines during pyrosequencing. During this process DNA is fragmented, which can lead to chimeric products and interfere with sequencing of long sequences. Moreover, genome complexity is reduced to three nucleotides, which underlies the requirement for longer primers and may be an issue for sequence alignment in larger studies. Importantly, if conversion of unmethylated cytosines is incomplete, this can lead to misleading quantification of DNA methylation.

Peg3 belongs to a small group of 100-200 autosomal genes that are regulated by genomic imprinting in mammals, with DNA methylation permanently silencing one of the parental alleles. Specifically, *Peg3* is maternally imprinted and paternally expressed (1297). *Peg3* DNA methylation has been studied more extensively in the mouse compared to the rat. At the time of the experiment, a literature search found only assays designed for the mouse genome (1297, 1309). After the experiment, one published assay for the rat genome was found (1310).

In this pilot study, *Peg3* methylation was found to be very close to the expected 50% value for all CpG sites assessed. This provided evidence that bisulfite conversion of DNA successfully discriminated between methylated and unmethylated cytosines, and pyrosequencing successfully sequenced these cytosines. Since the observed DNA methylation values are close to the true methylation values and there was little variability in the measurements, it can be reasonably concluded that the methodology had good accuracy and precision (bisulfite conversion, two-step PCR, pyrosequencing, analysis). This novel pyrosequencing assay will be valuable in future studies of *Peg3* methylation in the rat genome, including studies requiring an internal control for the methodology.

4.5.2 There is no evidence that DNA methylation in the promoter of Glt1 is affected by hypoxia-ischaemia

Briefly, there was no evidence that HI altered DNA methylation in any of the candidate regions in *Glt1* promoter in the ipsilateral cortex, other than very small (<5%) effects of dubious biological relevance.

CpG islands are nearly always unmethylated (939, 968-972), with few exceptions (e.g. imprinted genes) (971). The *Glt1* CpG island is indeed unmethylated in the P1 rat cortex and cerebellum, with established inducers of *Glt1* leaving DNA methylation unaffected (1207, 1208). On the other hand, hypermethylation of the *EAAT2* CpG island has been previously reported in human glioma cell lines, with *EAAT2* silencing (1311). In this study, the CpG island of the *Glt1* promoter was found to be unmethylated in the P7 cortex, with no evidence of significant changes after HI.

In humans, the region homologous to the proximal promoter was identified as differentially methylated in saliva-based EWAS studies of very preterm vs term newborns (1292) and term-born infants at 6 weeks of life vs 1 year (1312), potentially reflecting changes induced by the profound stress of prematurity but also age-dependent changes in methylation.

Multiple CpG sites across the *EAAT2* CpG island and proximal promoter have also been reported in EWAS of bipolar disorder and addiction, coinciding with regions near consensus sequences for transcription factors (1313). In this pilot study, there was only evidence of a very small time effect in the proximal rat promoter, with 1% higher methylation at 24h vs 6h in all groups. There was also a very small effect in blood, whereby methylation was approximately 3% higher in HI rats vs naïve and sham but only at 6h.

In epigenome-wide association studies (EWAS), it has been suggested that more confidence can be held in the biological relevance of effects larger than 10%, whereas effects smaller than 5% should be interpreted with great caution (1314, 1315). This is a small hypothesis-driven pilot study with limited sample size and using bulk tissue with multiple cell types; due to the high cost of false negatives in exploratory studies, it can be argued that more subtle changes may be of interest and guide a follow-up experiment. However, these effects are very small and do not overcome the 5% detection limit of pyrosequencing (1306, 1316). Additionally, the change in blood disappeared after 6h and was not correlated to changes in the cortex. To my knowledge, there is no published evidence regarding DNA methylation in this region in the rat brain. A region further downstream in the rat proximal promoter (~-900 bp) was shown to be unmethylated in P1 cortical astrocytes similar to the CpG island closer to the TSS (1208). This region was not followed up by the authors in subsequent work (1207).

Shore regions are sequences located up to 2 kb from the TSS and outside typical CpG island promoters. They are characterised by lower CpG density, and indeed only 2 CpG sites could be assessed by bisulfite pyrosequencing in this study. DNA methylation is typically more dynamic in these regions and varies in a tissue- and region-specific way (974, 975, 1317, 1318). The function of methylation in these regions is less clear: the correlation with transcriptional silencing is not well understood and it is not known whether it is a cause or a consequence of gene silencing or what other implications it may have (974, 976, 977). Other epigenetically regulated genes have CpG islands that are protected, while differential methylation occurs at more distal enhancer elements with methylation-dependent function (1319). Accordingly, the *Glt1* shore region has been shown to have more dynamic DNA methylation patterns compared to the CpG island, responsible for differences in gene expression in different brain regions (cortex vs cerebellum) both at baseline and in response to established inducers of *Glt1* (1207, 1208). This higher epigenetic plasticity allows the region to act as an epigenetically-regulated enhancer in the cortex, as confirmed by a reporter

gene study in rat astrocytes (1207, 1208). In this pilot study, there was no evidence that DNA methylation at the distal shore region was affected by HI. There was some evidence of a very small effect of anaesthesia, with the sham group showing approximately 4% lower methylation than the naïve group independently of time. Once again, the effect size is small and of dubious biological relevance and was not associated with any transcriptional change within the 24h. However, it would be worth following up this finding, perhaps in the context of larger analyses, since there is evidence that isoflurane can be both neuroprotective or neurotoxic (depending on duration of exposure) and it affects glutamate signalling (see 5.2.2). Follow-up may be achieved by analysing DNA methylation in isolated astrocytes and including a later time point for transcriptional analysis (e.g. 72h).

Crucially, the good quality *Peg3* data suggests that a lack of significant DNA methylation changes for *Glt1* reflects true negative findings with these experimental conditions and sample size rather than a technical failure to detect effects.

Epigenetic mechanisms other than DNA methylation were not assessed in this pilot study. However, epigenetic mechanisms do not act independently but in concert with each other to alter chromatin structure and accessibility for transcription and translation (1320-1324). For example, synthetic corticosteroid dexamethasone induces *Glt1* in the cortex and increases histone acetylation in the shore region in the absence of DNA methylation changes (1207). Compared to the relative stability of DNA methylation, histone modifications have a highly transient nature and can be thought as reflecting the dynamic regulation of transcription rather than long-term gene silencing (927). Perhaps the effects of the environmental stress of HI are reflected in more dynamic and transient histone modifications, which may be assessed at candidate genes with chromatin immunoprecipitation followed by PCR (1325).

4.5.3 Strengths and limitations

A strength of this study was inclusion of the *Peg3* methylation data, which allowed more confident interpretation of these findings as true negatives with current experimental conditions and sample size. Assessment of both brain and blood DNA methylation added clinical relevance to the study, enabling assessment of DNA methylation as a potential biomarker in newborns.

Main limitations of the study include limited sample size and power. This issue may be exacerbated by the use of bulk tissue, where differential methylation across cell types may prevent detection of effects in individual cell populations (e.g. astrocytes). Moreover, the

study did not assess DNA methylation changes beyond 24h or in other vulnerable brain regions (e.g. hippocampus, thalamus). DNA methylation changes at other regions of the *Glt1* promoter and the cytokine promoters could not be evaluated, due to time and funding restrictions. Finally, histone modifications were not evaluated in this study and may represent a more dynamic epigenetic mechanism sensitive to the environmental stress of HI.

4.6 Conclusion

There was no significant evidence of biologically meaningful DNA methylation changes at the *Glt1* promoter in the cortex of P7 rats in the 24h following HI. This aligned with the lack of obvious transcriptional changes. The very weak non-significant evidence of lower expression in HI vs sham in primary analyses, which strengthened in secondary analyses merging naïve and sham groups, may be an artefact. It is not possible to assess this without increasing sample size and/or assessing DNA methylation and transcription of *Glt1* in astrocytes in isolation. Assessing DNA methylation changes at the cytokine promoters remains of great interest given evidence of upregulation in the acute phases after HI in this pilot study and the wider literature.

5 General discussion

This project aimed to evaluate genetic and environmental influences on regulation of glutamate transport and inflammation in relation to two of the most clinically relevant perinatal brain injuries (cPVL and HIE) and subsequent motor and cognitive impairment in childhood. The project integrated epidemiological approaches in newborns and experimental approaches in animal models. Genetic, epigenetic, and transcriptional regulation was assessed for four candidate genes, i.e. the main glutamate transporter in the brain (*EAAT2*/*Glt1*) and three key pro-inflammatory cytokines (*TNFα*, *IL1β* and *IL6*). Table 5.1 contains a summary of the research questions and main findings of the project. In the general discussion, relevant genetic, epigenetic, and transcriptional approaches in newborns and in animal models will be discussed.

Table 5.1. Research questions and main findings of this project

Chapter	Research Question	Main Findings
2: SNP association study in very preterm newborns	Are regulatory variants in the 4 candidate genes associated with risk of cPVL, motor and cognitive impairment in children born very preterm?	• Replicated previous evidence of association between *TNFα* -308 in children born very preterm with cPVL and CP • First evidence of association between *IL1β* -511 and the British Ability Scales score assessing cognitive skills at 5 years of age, in particular the non-verbal reasoning subscale • Suggestive evidence of association between *IL6* -174 and any moderate to severe white matter injury at birth • Suggestive evidence of association between the linked *EAAT2* -200 and -181 variants and the verbal skills subscale of the British Ability Scales at 5 years of age
3: Gene expression study in a rat model of hypoxic-ischemic brain injury at term	Is transcription of the 4 candidate genes altered in the cortex and hippocampus in the 24h after acute hypoxia-ischaemia?	• Hypoxia-ischaemia causes an early neuroinflammatory response in both regions with upregulation of all 3 cytokines • Hypoxia-ischaemia also causes changes in brain injury markers, signalling onset of astrogliosis and myelin injury • Hypoxia-ischaemia may cause a small loss in *Glt1* in the cortex and/or isoflurane may cause a small increase in *Glt1*
4: DNA methylation study in a rat model of hypoxic-ischemic brain injury at term	Is DNA methylation at the promoter of 2 of the 4 candidate genes (*Glt1* and *Tnfα*) altered in the cortex and blood following acute hypoxia-ischaemia?	• Hypoxia-ischaemia does not cause any DNA methylation changes at 3 candidate promoter regions of *Glt1* • (*Tnfα* could not be assessed due to COVID-19-related disruptions)

5.1 Genomic studies of cerebral palsy and newborn brain injuries

If the genetic landscape of CP reveals itself to be similar to that of other neurodevelopmental disorders, multiple genes and types of genetic variants will be involved, e.g. SNPs, indels, rare single-base variants and large structural variations that are individually rare but cumulatively common (875-877, 1326-1328). Integration of different genetic technologies

(e.g. microarray, sequencing, *in silico* analyses), designs (candidate vs hypothesis-free approach) and outcomes (e.g. molecular, neuroimaging and disease outcomes) will be required to advance our understanding of the genetic architecture of CP. Moreover, integrating haplotype analyses alongside single-SNP analyses will allow a more in depth understanding of genetic effects.

5.1.1 Understanding genetic effects: from single SNP to haplotype analyses

Single SNP association studies do not consider the genetic sequence surrounding the variant under study. Moreover, they do not consider the diploid nature of the genome, since they do not separate, or phase, alleles inherited from the mother and father (1329). An alternative way to analyse genetic associations is by haplotype-based analyses (1330). These carry more biological relevance since genetic variants are not inherited independently of one another but in haplotype structures based on linkage disequilibrium (LD). A haplotype is a set of genetic loci located on the same chromosome and co-inherited from a single parent. These co-inherited variants are in LD often, but not exclusively, through physical proximity along the linear genome. New haplotypes are born from mutations arising and being passed on in the population, and are destroyed by meiotic recombination, with population genetic forces acting as modifying factors (1331). Hence, SNPs identified by genetic association studies may simply tag linked variant on the same haplotype. There may also be multiple functional variants on a haplotype jointly acting as a "super allele" with a larger effect on a trait (e.g. multiple variants affecting folding of proteins) (1330, 1332, 1333).

Importantly, haplotype effects may underlie the commonly observed "flip-flop" phenomenon, whereby the direction of effect flips from protective to detrimental or vice versa in replication studies (1334, 1335). This was also observed when comparing our findings to the wider literature and does not invalidate findings. On the contrary, evidence from multiple independent samples supports evidence of genetic association, with the flip-flop effect pointing to potential population differences in underlying allele and haplotype structures and frequencies. This is also seen for functional studies, with higher expression reported with both *IL6* -174 G allele (1053, 1056, 1058, 1336-1338) and *IL6* -174 C allele (1059, 1339-1342), *IL1β* -511 T allele (1052, 1343, 1344) and C allele (1345-1347). For example, the finding from Fishman and colleagues (1053) that the C allele was associated with lower plasma IL6 levels in healthy subjects may be due to the fact that the low-expression haplotypes containing the G allele were rare in the population. Of course, factors other than

haplotypes affect results from reporter gene assays, including choice of cell and inclusion of other regulatory sequences (e.g. 3' UTR) (1348).

Haplotype-based analyses should be a priority in the future, supported by the substantial evidence that haplotype effects govern cytokine expression *in vitro*. This has been extensively studied with reporter gene assays for variants in the *IL1β* promoter (1052). *IL1β* -511 induces only a modest upregulation in *IL1β* when considered as an individual SNP, whereas upregulation is strong when the -31 variant is also present. Moreover, inclusion of two other variants (-1464 and -3737) substantially suppresses IL1β only if the minor allele is present at both -511 and -31. Interestingly, the highest expression haplotype (-1464G/-511T/-31C) is the least common in Caucasians (6%) but over 7-fold more common in African-Americans (46%). When assessing haplotype effects in gingival fluid specifically from a Caucasian sample (1346), it was found that three out of four pro-inflammatory haplotypes, present in over half of the sample and associated with 28-52% higher IL1β levels, featured *IL1β* -511 CC genotype rather than the minor T allele, and the fourth included the CT genotype. We found that children with *IL1β* -511 CC genotype have lower BAS general conceptual ability scores. The CC genotype has also been reported in association with increased risk of myocardial infarction and ischemic stroke in young patients, as well as higher levels of IL1β in LPS-stimulated blood cells (1347, 1349). Importantly, experimental evidence shows that variants other than -511 in the *IL1β* promoter (e.g. -31 or -3737) alter transcription factor binding (1052, 1350, 1351), suggesting that this variant may simply tag a non-genotyped functional variant.

Similarly, other SNPs in the *TNFα* promoter (e.g. -857) have been shown to be functional (1352). Moreover, *TNFα* -308 may simply tag neighbouring causal genes in the MHC region (e.g. *HLA* antigens, *TNFβ*, *CD14*) (1034, 1121). For example, the variant belongs to a haplotype including a variant in *TNFβ* which alters expression of TNFβ (1353). This cytokine is involved in adult stroke and autoimmune disorders (1354-1356), and levels in serum are associated with gross motor impairment in school-aged children with neonatal encephalopathy (652). Importantly, etanercept, which has been used as anti-TNFα therapy in rodent models of newborn brain injury, also blocks TNFβ (1357).

Haplotype effects on gene expression and relevant outcomes have also been shown for *IL6* (1058, 1358-1362). *IL6* -174 belongs to a 7-SNP haplotype, with homozygotes at the entire haplotype having a higher risk of CP than homozygotes at any individual variant (918).

Importantly, *IL6* -174 may tag a variant in intron 4 (+3331) disrupting a CpG methylation site (1363) and/or a string of As and Ts (-373AnTn) which may affect the 3D structure of DNA (1358). Haplotype effects on *EAAT2* expression *in vitro* have not been assessed, and very few studies exist assessing haplotype effects on disease risk (e.g. schizophrenia) (1050).

As well as carrying more biological relevance, haplotype-based analyses have been shown to improve power and robustness of genetic association studies of complex diseases (1330, 1364, 1365). The main limitation is that haplotype phasing information is rarely available, including in the current study, and collection requires a substantial effort (1333). Experimental phasing is expensive and labour-intensive and tends to be limited to studies where accurate long-range haplotypes are needed and family data is not available (1366, 1367). Computational estimation of haplotypes in unrelated individuals is cheaper and faster but requires knowledge of the LD patterns in the sequence surrounding the variant, which was not available for the current study. This information is used to estimate the probability of any given variant belonging to a set of possible haplotypes based on observed genotype frequencies (1367).

In future genetic studies of newborn brain injuries and neurodevelopmental outcomes, haplotype analyses could be carried out by genotyping larger regions (e.g. 500 bp on each side of the variant) or estimating haplotype frequencies in a more widely genotyped reference population (e.g. ALSPAC) (see 5.1.2).

5.1.2 Choice of study design: from candidate gene to genome-wide approaches

Follow-up work to this project will involve assessing a larger selection of candidate inflammatory and glutamatergic variants in relation to a variety of neonatal and childhood adverse motor, cognitive, educational, and behavioural outcomes in the Bristol-based ALSPAC birth cohort (also known as “The Children of the 90s” cohort). This cohort provides a unique opportunity to validate and add to these findings in a large sample with genetic, epigenetic and phenotypic data collected longitudinally from birth to childhood (1368). Availability of parental data additionally offers opportunities to analyse parental-offspring gene-gene and gene-environment interactions, estimate haplotypes more accurately, and identify possible sources of bias in genetic studies with unrelated individuals. Information on LD patterns will also allow identification of SNPs tagging the EAAT2 SNPs, which are not present on any of the widely used lllumina microarrays and are therefore not directly genotyped in any GWAS studies. Epigenetic data is available for the ARIES subset of

ALSPAC, allowing exploration of genetic effects on DNA methylation (mQTL effects) (1369).

For common variants, the candidate approach still represent the main genetic epidemiology approach in the field. The approach benefits from building on previous scientific evidence for formulation of the *a priori* hypothesis, however this can also be a limitation, since our understanding of disease pathophysiology is often limited. SNPs in other inflammatory genes have been implicated in CP in preterm newborns through a candidate approach (e.g. *IL8*, *IL10*, *IL18*, *TNFβ*, *COX1*, *COX2*, *MBL*, *TLR4*) (162, 654, 901, 906, 907, 910, 917, 1027, 1115, 1370, 1371). Moreover, SNPs in other pathways have been involved in preterm brain injury, including developmental pathways (e.g. *WNT/β-catenin*) (1372), neuronal migration and lipid metabolism (1373). Importantly, some of the genes involved in newborn brain injuries and CP may not even be currently suspected and allowing for wider analyses beyond the traditionally studied genes (e.g. cytokines) will be key (1374). This was recently demonstrated by a study integrating human and mouse molecular and neuroimaging data at the genome level, which reported a role for the less known microglial DLG4 protein in preterm white matter injury (1279).

GWAS of relevant perinatal data have only recently started to accumulate, including preterm birth, gestational duration, clinical chorioamnionitis, bronchopulmonary dysplasia, necrotising enterocolitis and growth restriction (1375-1382). Interestingly, fetal variants in pro-inflammatory cytokines (including IL1 family) emerged as associated with gestational duration (1383). The main barrier to GWAS studies of newborn brain injuries or the polygenic forms of CP is currently the lack of sufficiently large samples held by any individual research centre, especially if focusing on specific subpopulations (e.g. very preterm). The issue of sample size has been widely illustrated in the field of psychiatric disorder research, where GWAS have been used for the last 15 years (1384), with only a handful of variants identified for schizophrenia with samples in the order of hundreds (1385), raising to over 100 SNPs with a sample in the order of tens of thousands (1386). National and international collaborations such as the International Cerebral Palsy Genomics Consortium formed in 2017 will be paramount in achieving adequate sample sizes (839). In England, a further issue is represented by the lack of a national CP register and valuable approaches may involve machine learning methods identifying likely cases of CP based on linkage of prescription data, medical records and secondary care records (1387, 1388).

Insights gained from an initial GWAS of CP could help deciding whether to pursue whole exome and/or whole genome sequencing to uncover rare variants. For many complex traits, a combination of all three approaches (microarrays, WES and WGS) will be required, to identify associated genetic variants with a range of effect sizes and frequencies (858). Bioinformatic tools (e.g. UCSC Genome Browser, Ensembl Variant Effect Predictor, Polyphen2 software for coding non-synonymous variants) can help predict functional effects and support identification of true causal variants before proceeding to lab-based functional studies (1023, 1389-1392). RegulomeDB and HaploReg combine information from ENCODE and the Roadmap Epigenomics Project and can be useful to prioritise GWAS hits that may be causal to disease by affecting gene expression. RegulomeDB provides information on transcription factor binding sites, cell-specific chromatin states and eQTL data (1393). HaploReg increases the chance to identify causal variants that may be in LD with the query variant by estimating haplotypes using the 1000 Genomes Project population data (1394).

Once robust GWAS data will start accumulating, more advanced genetic epidemiology approaches will become available, including Mendelian randomisation (MR) and polygenic risk scores which may be used for earlier identification of high risk newborns (1395). In MR approaches, genetic variants robustly associated with an environmental risk factor are used as proxies to evaluate whether the environmental risk factor has a causal effect on disease. These approaches exploit the random inheritance of genetic variants at conception, resembling random assignment to treatment arm in an RCT, conferring robustness to the issues of confounding and reverse causation (1184, 1396-1398). MR approaches have recently been used to assess the Barker's hypothesis of developmental origins of disease, e.g. to assess whether the observational association between birth weight and a range of adult diseases is causal (1399, 1400). Several studies have reported a causal association between birth weight and cardiovascular diseases and type 2 diabetes (1401-1403), while the causal relationship with psychiatric disorders has been proposed to be due to confounders or small effect size (1404). MR approaches are relatively new and constantly evolving, including strategies to account for the interaction between parental and foetal genotypes, and are likely to become increasingly used in epidemiology (1405-1408). Chorioamnionitis represents an interesting example of a poorly understood pathophysiology which could benefit from this approach (397). While there is strong evidence of an association between chorioamnionitis and motor (including CP), cognitive and behavioural impairment (239, 387, 389, 390, 392,

393, 401, 414-419, 630, 1409-1414), it is not clear whether the effects on perinatal brain injuries are causal or due to other perinatal factors (e.g. preterm birth, z-scores) (395, 397, 1415-1419). MR approaches could also help with prioritising targets for neuroprotective drugs or repurposing existing drugs before expensive trials are conducted. This has been recently shown in a study evaluating pharmacological blockade of the IL6 receptor for coronary heart disease (1420). Specifically, SNPs robustly associated with increased IL6 gene expression were used to generate groups differing only by IL6 levels (which is compatible with pharmacological IL6 receptor blockade), allowing comparisons of disease outcomes and distinction between on- and off-target effects.

5.1.3 Choice of outcomes: integrating short-term and long-term outcomes

Heritability has classically been measured in relation to disease outcomes. More recently, technology developments have expanded the range of phenotypes that can be explored to molecular phenotypes (e.g. gene expression, metabolites measured by mass spectrometry) and neuroimaging phenotypes (e.g. MRI brain scans). This broadens the insights that can be gained through genetic studies by shifting the focus to the core biological processes that may in turn impact disease risk itself. This is important, for example, for drug development as drugs act on protein targets and biological processes to create a change in phenotype and protect from disease. Inclusion of neuroimaging outcomes will allow exploration of any genetic effects on brain structure and function in the perinatal period, which may mediate later neurodevelopmental impairment. In terms of neuroimaging-genetic approaches, a key advancement in the field will be the creation of a biomedical resource linking and harmonising hundreds of neonatal MRI scans between each other and with genetic data. An example from adult studies is the Enhancing Neuroimaging Genetics through Meta-Analysis (ENIGMA) Consortium, a collaboration from 70 institutions worldwide which has allowed the first GWAS studies of adult brain volumes (1421, 1422).

Even more proximal outcomes can be chosen, such as gene expression. A GWAS including over 8,000 Finns recently identified 27 SNPs associated with circulating levels of one or more cytokines, including 15 SNPs affecting gene expression (eQTL effects) in whole blood (1423). Three SNPs were linked to both gene expression and protein, supporting candidacy as drug targets in adult immune diseases. Neither of the variants studied here were amongst these genome-wide hits, although variants in genes interacting with these cytokines were identified (e.g. TNF-related apoptosis inducing ligand). It would be of great interest to carry

out a similar GWAS in neonatal blood, including at baseline and after *ex vivo* immune challenge (1424).

5.1.4 Translational value of genetic studies

Investigating the genetic contribution to disorders with clear environmental risk factors, such as newborn brain injuries and neurodevelopmental disorders including CP, is driven by three main motivations. Firstly, it can advance our understanding of the biological mechanisms of disease, both by identifying the key mediators and pathways leading to disease, and by offering a tool to explore the causal effects of environmental risk factors themselves (Mendelian randomisation).

Secondly, drug targets with genetic support are twice as likely to succeed and genetic evidence is the second biggest predictor of drug success (after repurposing of drugs already in use) (1425). Even if the individual SNP effect is small, robust associations can point to attractive protein targets if manipulation of the protein itself has a large effect on disease. Genetic evidence can also help excluding unattractive drug targets, e.g. when manipulation of the protein has no effect on disease despite a large effect on protein levels. pQTL Mendelian randomisation approaches are emerging as particularly valuable for drug target discovery: SNP effects on protein levels (pQTL effects) robustly identified by GWAS can be exploited in MR approaches to evaluate the causal effects of such proteins on disease (1426).

Thirdly, knowledge of genetic risk can aid in risk prediction. As sample sizes increase and genetic variants are uncovered, the proportion of trait variance explained by genetic factors will also increase, improving the diagnostic power for polygenic forms of CP. Importantly, even if the genetic component is small compared to the environmental component, the clinical value could be substantial if it leads to meaningful differences in clinical management (e.g. earlier identification, diagnosis and treatment, and more tailored approaches). Genetic risk prediction can also aid researchers in the designs of clinical trials. Approximately half of phase 3 trials fail (1427-1429), and incorporating genetic information to identify high-risk individuals has been proposed as a way to reduce trial cost and duration (1430).

Finally, pharmacogenetics is an area that holds promise of a powerful impact in clinical management of perinatal brain injuries. Genetic background is one of the factors contributing to differences in drug responsiveness and adverse reactions (1431), since it can affect pharmacokinetics (e.g. variants affecting drug bioavailability) and pharmacodynamics (i.e.

variants affecting drug targets). Accordingly, it was recently shown that treatment with magnesium sulphate abrogated the detrimental associations between inflammatory SNPs (e.g. *Il1β*, *IL6*, *MBL2*) and adverse neurodevelopmental outcomes in children born preterm (919). Identifying the best dose of immunomodulatory therapies currently being developed may be essential in the future given the potential adverse effects. To this end, neonatal neuroprotection research may draw knowledge from studies of the adult stroke drug warfarin. Here, pharmacogenomic approaches have been key since patients with certain genetic variants require lower doses to avoid fatal bleeds (1431), and ongoing clinical trials are evaluating genotype-guided dosing. Technological advances now include handheld devices which genotype DNA in minutes and hold great promise for rapid pharmacogenetic profiling and a push toward a 'right first time prescribing' approach (1432, 1433).

5.2 Epigenetic and transcriptional studies of hypoxic-ischemic brain injury

A modified Rice Vannucci model was used to model HI brain injury in the term newborn, with left carotid artery ligation followed by 30 min recovery and 100 min hypoxia at 8% oxygen at 36°C, instead of the original 3.5 h of 8% oxygen at 37°C (1196). These parameters have been previously shown to produce moderate brain injury with around 40% brain tissue loss in the ipsilateral hemisphere relative to the contralateral hemisphere one week after HI in the P7 rat (1200). Injury involves necrosis of neurons and glial cells, microgliosis and astrogliosis typically observed in the cortex, basal ganglia, hippocampus, subcortical and periventricular white matter, and reflects patterns observed in HIE (1196).

5.2.1 Neuroinflammation in hypoxic-ischemic brain injury

5.2.1.1 Likely cellular sources and targets of cytokines

In this pilot study, three key pro-inflammatory cytokines were found to be upregulated at the transcriptional level in the ipsilateral cortex and hippocampus. Based on previous evidence, the main cellular sources of cytokines following HI are likely to be activated microglia, infiltrating peripheral immune cells (e.g. mast cells, monocytes/macrophages, neutrophils) and reactive astrocytes (375, 516, 807).

Microglial activation has been demonstrated in the post-mortem hippocampus of newborns with HIE (1434). Following HI, cells damaged by excitotoxicity release danger associated molecular patterns (DAMPs) in the microenvironment (375, 805). DAMPs start activating microglia as early as 2-4h post-HI in the newborn rat brain (e.g. via IL1 receptor, Toll-like

receptors, cytoplasmic NOD-like receptors) (1221, 1222, 1244, 1435-1437). Activation involves changes in morphology, proliferation rate, gene expression and acquirement of new functions, including phagocytosis of dying neurons (514, 516, 1438-1440). Activated microglia can damage the developing neurons, not only by losing their homeostatic and developmental functions, but also by releasing pro-inflammatory cytokines, reactive oxygen species, death receptor agonists and glutamate, potentiating further release by astrocytes and contributing to disruption of the blood brain barrier (375, 512, 514, 573, 813, 1441-1444). NFκB seems to be key in this process, with different signalling pathways converging on upregulation of this mediator leading to cytokine release (98, 375, 480, 1445-1455).

The increased permeability of the blood brain barrier caused by HI allows peripheral immune cells to infiltrate from the cerebral blood vessels into the brain (375, 1456). Activated microglia and astrocytes release chemokines (e.g. MIP1α), which further attract peripheral immune cells to the area of ischemic injury (1221, 1457, 1458). Infiltration of myeloid cells peaks around 24h post-HI and involves mainly neutrophils, dendritic cells, and monocytes, which give rise to local macrophages with phagocytic and antigen-presenting properties (375, 1459). Mast cells, which bridge innate and adaptive immunity, can not only infiltrate but also proliferate locally for weeks after injury, releasing increasing levels of pro-inflammatory cytokines, such as TNFα (375, 516, 1460, 1461). HI can also cause local activation of the complement system and antimicrobial peptides (1462). Moreover, HI can cause infiltration of antigen-presenting cells and T lymphocytes, which are found in the ipsilateral hemisphere months after HI (375, 1463). The roles of these peripheral cells and mediators in the injury process are subject of current research, including the chronic component of neuroinflammation and potential interference with repair mechanisms (375, 516, 804, 1221, 1223, 1464).

Reactive astrocytes represent an additional source of pro-inflammatory cytokines. Both astrogliosis and microgliosis have been reported to start as early as 3h post-HI in the injured hippocampus and corpus callosum, with microgliosis peaking in the first week and decreasing from day 14, and astrogliosis peaking at 14 days, with the glial scar still evident at day 100 (1223). Reactive astrocytes are thought to have dual neuroprotective and neurotoxic roles which are only partially understood, with the relative contribution probably depending on severity of the insult and time of recovery (516, 1465, 1466). Alongside allowing scar formation, they can promote anti-inflammatory mechanisms and dedifferentiate to newly available neural progenitor cells (1467). However, reactive astrocytes also contribute to the

HI injury process, not only by losing their homeostatic and developmental functions but also by becoming themselves sources of pro-inflammatory cytokines, glutamate, and reactive oxygen species (516, 738, 1216, 1256, 1468-1472). In the rat brain, reactive astrocytes have been shown to be both cellular sources and targets of cytokines (including TNFα and IL6). Inhibiting cytokines, for example by hypothermia, reduces not only neuronal injury but also astrogliosis (483, 1473).

This pilot study was limited by the inability to identify not only cellular sources but also the cellular targets of the cytokines, which are likely to include glial cells, neurons, peripheral immune cells, and endothelial cells (516, 1474). Cytokines may contribute to brain injury via multiple mechanisms, including disruption of the cerebral blood flow oxygen delivery due to their vasoactive properties; direct neuronal and astrocyte injury due to their cytotoxic effects; and potentiation of glutamate excitotoxicity, inflammation and oxidative stress in complex interactions of glial cells and neurons with vicious circles of cytokine and glutamate release (101, 532-534, 549, 552, 1475, 1476). With regard to potentiation of glutamate excitotoxicity, the specific mechanisms are largely unresolved, but they are thought to converge on accumulation of extracellular glutamate (e.g. via impaired astrocytic uptake and increased glutamate release by activated glial cells) and overactivation of glutamate receptors (e.g. via accumulation of extracellular glutamate, alteration of number and chemical composition of neuronal glutamate receptors and expression of glutamate receptors by reactive astrocytes) (494, 585, 811-813, 1477, 1478).

Given the evidence of transcriptional changes following HI, assessment of epigenetic regulation of these cytokines in this Rice Vannucci model is of great interest. This may be achieved by serial bisulfite pyrosequencing as done here, with a higher number of tandem assays along the amplicon if required (1316). An even more comprehensive analysis could be carried out with the PacBio sequencer, which allows accurate sequencing of long-reads beyond 20 kb. This would enable detection of DNA methylation across the entire gene, including promoters, distal shores, gene bodies and 3'UTRs (974).

Overall, preclinical evidence supports the notion that HI triggers an early inflammatory response which contributes to injury of neurons, axons, and oligodendrocytes depending on stage of brain development (1463, 1479, 1480). In humans, evidence of a causal relationship between HI and inflammation comes from observation that cerebral ischaemia induced by circulatory arrest during cardiac surgery in the newborn triggers a systemic inflammatory

response within minutes (1480). Existing evidence supports the concept of stratification of HIE newborns by inflammatory status at birth and further research into immunomodulatory therapies for neuroprotection (485, 557, 559, 1481).

5.2.1.2 Targeting inflammation for neuroprotection of the vulnerable newborn

The mechanisms through which therapeutic hypothermia exerts neuroprotection are not fully understood, but are thought to include protection from glutamate excitotoxicity, inflammation, oxidative stress, and apoptosis (723, 1482). Hypothermia lowers cytokine levels in serum and CSF, and peripheral cytokine levels correlate with MRS-defined injury (341). Unsurprisingly, clinical studies assessing how cytokine levels are affected by hypothermia are starting to accumulate (640, 1265, 1483, 1484).

Evidence of an early neuroinflammatory response has triggered interest in immunomodulatory therapies targeting cytokines directly. The most convincing evidence currently supports IL1 receptor blockade as a therapeutic approach, based on the neuroprotective effects seen with pharmacological or genetic inhibition in newborn rodents (555-563). Safety trials are required to assess the potential issue of broad immunosuppression observed in adults (1485). Cytokines have pleiotropic effects on a variety of targets and systems, including important homeostatic, developmental, growth and repair functions (1444, 1486). Accordingly, genetic or pharmacological inhibition of TNFα after an acute HI insult or a combined inflammatory/excitotoxic insult is neuroprotective, reducing brain injury by half in some studies (485, 568-570), however mice lacking TNFα receptors are more susceptible to HI injury (576). In the preterm mouse, inflammatory genes are upregulated in pre-oligodendrocytes as part of developmental programmes, suggesting that global suppression may not be a safe and effective neuroprotective strategy in the preterm newborn (1487). How different the scenario is for the more developmentally mature term brain is not known. Targeting multiple transcription factors at low doses and selectively blocking individual pathways (e.g. apoptotic but not anti-apoptotic TNF receptor pathway) might be better approaches than globally suppressing the inflammatory response. Local delivery to the brain might also be a safer and more desirable approach, e.g. via perispinal administration, engineered fusion proteins small enough to cross the blood brain barrier (as shown for the TNFα receptor) (1488, 1489), or intranasal administration of inhibitory peptides (as shown for NFκB) (1490). The effectiveness and safety of pharmacological cytokine modulation remains controversial even in adult models with more data available than for HIE (1444), and more neonatal data is needed.

5.2.2 Glutamate transporter regulation following hypoxia-ischaemia remains worth further attention

In this pilot study, there was only very weak non-significant evidence of lower *Glt1* expression in the cortex in the HI group compared to the sham group, which strengthened in secondary analyses merging naïve and sham groups. There was no evidence that DNA methylation changes in the three candidate promoter regions preceded this potential transcriptional change. The lack of significant evidence of DNA methylation and transcriptional changes with these experimental conditions does not currently support follow-up protein analyses for GLT1. This negative evidence does not align with previous *in vitro* and *in vivo* evidence of GLT1 dysregulation following hypoxia and hypoxia-ischaemia.

GLT1 (both mRNA and protein) is lost in the adult rat cortex and hippocampus after ischaemia (1491-1494). GLT1 protein is also downregulated in astrocyte cultures from P6-8 rats subjected to hypoxia (via NFκB) (1495). To my knowledge, only one study exists assessing GLT1 expression in the P7 rat, using similar but not identical experimental conditions (left carotid artery ligation, 2h recovery and 2h 8% hypoxia at 33°C) (1240). Here, GLT1 protein was lost together with GFAP and MAP2 in the injured cortex and hippocampus at 12h. Both GLT1 and GFAP recovered by 48h in the hippocampus but not the cortex. On the other hand, GLT1 was unchanged in the border zone, where GFAP increased signalling astrogliosis. The study was limited by very small sample size (e.g. 2 rats in the 12h group destined to histological analyses). Moreover, the authors show only micrographs at 24h despite reporting loss of GLT1 at 12h. Nonetheless, the study supports a loss of GLT1 at the protein level following HI. In the current study, *Gfap* was upregulated and not lost in both cortex and hippocampus between 6h and 24h. This aligns with the notion that astrogliosis is an early response to HI in the rodent brain (483, 1220-1222), whereas it seems to be an adaptive mechanism to the acute phases in the piglet brain (1496-1498). Perhaps the slightly longer duration of hypoxia, recovery period between ischaemia and hypoxia, or different temperature control contributed to more severe injury in the study by Fukamachi and colleagues. This may have led to astrocyte abnormalities with an initial loss of both GFAP and GLT1 in the ischemic core which did not occur in our study. Alternatively, loss of GLT1 and/or GFAP in our pilot study may have occurred post-translationally and/or locally in the ischemic core but remained undetected when assessing total lysates from cortex and hippocampus, which do not differentiate between ischemic core, border zone and uninjured tissue.

GLT1 loss following HI has also been shown in the newborn piglet. Consistent with impairment of glutamate transport, extracellular glutamate concentration becomes elevated in the cortex, basal ganglia, and thalamus after hypoxia (1499). Acute HI causes neuronal injury in the piglet striatum, with initial simultaneous loss of astrocytic GLT1 and GFAP proteins at 24h and recovery of GFAP but not astrocytic GLT1 by 96h (1497). The gradual near complete loss of astrocytic GLT1 in the injured regions was accompanied by upregulation in neurons, suggesting a compensatory mechanism in response to increasing extracellular glutamate levels. In a piglet model of severe hypoxia, loss of astrocytic GLT1 started as early as 6h and was extensive by 72h (1500). At the same time, the GLT1B splice variant, normally expressed by astrocytes (1501), was upregulated in neurons by 72h. Crucially, astrocytic GLT1 was lost only in the injured regions, including cortex and hippocampus but not the dentate gyrus, supporting a role in the injury process via glutamate excitotoxicity.

It is possible that baseline expression at P7 is too low to detect any transcriptional changes. GLT1 acquires astrocyte selectivity and undergoes profound developmental upregulation throughout the central nervous system from the second postnatal week onwards, reaching adult levels by weeks 4-5 (761-766). This may explain why knockout mice develop normally for the first few weeks and develop seizures and brain injury during postnatal week 3, with many dying by week 4 (760, 1502). This is also consistent with EAAT2 appearing in astrocytes at 41 weeks gestation in newborns and progressively increasing in all layers of the cortex in the first 1.5 years, at least in part via post-translational mechanisms (748). An interesting approach would be to repeat the experiment in P10 rats. Most studies with the Rice Vannucci model to date have focused on P7 rats, as the patterns of injury recapitulate the patterns seen in newborns with HIE, with injury in the cortex, thalamus, basal ganglia, hippocampus and subcortical white matter, and development of cortical cysts in longer survival models (79, 80). Working with P7 rats also allows to time the injury to coincide with the brain growth spurt and active myelination phase which signals the transition from preterm to term patterns of injury (356, 1503, 1504). Importantly, therapeutic hypothermia was developed with P7 rats and has been successfully translated to humans, and other neuroprotective treatments (e.g. xenon, melatonin, erythropoietin) have been studied in the same model (702, 1198, 1505, 1506). Nonetheless, the P10 rat brain is more similar to the full-term human brain in terms of myelination, neurotransmission, and brain electrical activity, and the Vannucci group recently replicated their traditional model in P10 rats

(1290). While the P7 Rice Vannucci model benefits from decades of published studies, the P10 model may be particularly relevant for studies of *Glt1*.

5.2.3 Choice of study design: from candidate gene to genome-wide approaches

5.2.3.1 Transcriptomic studies

Transcriptome-wide analyses have not been carried out in this rat model, however findings from a mouse model of HI injury at term have highlighted the value of hypothesis-free designs. Of the nearly 150 inflammatory genes (1257) and over 300 non-inflammatory genes (1507) found to be differentially expressed in the cortex, hippocampus, thalamus, and striatum between 2h and 72h post-HI, at least 90% had not been previously described in the newborn brain. The vast majority (97%) of the inflammatory genes were upregulated, generally from 8h and progressively increasing until the latest assessed time point at 72h (1257). This is in alignment with the early neuroinflammatory response found in the current study. The same pattern of early upregulation was observed for genes involved in transcription, stress, apoptosis, and growth. Genes involved in metabolism, cytoskeleton (e.g. *Gfap*), transport, enzymes and inhibitors were largely upregulated at a later stage (24-72h). Genes related to ion and vesicular transport, neurotransmission (e.g. *glutamate receptor ionotropic Nmda1*) and signal transduction were largely suppressed, and this suppression generally occurred at a later stage after HI (24-72h) (1507). Given this evidence of loss of *Nmda* glutamate receptor after 24h, it may be that excitotoxic shutdown at the synapsis and loss of glutamate transporter may have also been detectable after 24h.

With appropriate funding and resources, a hypothesis-free transcriptome wide study would provide great insight into global gene expression changes in this model. This could be designed as in a recent study with a rat model of combined inflammatory and HI insult (Hemmen Sebir, personal communication, manuscript in preparation), i.e. 1) by isolating a cell type of interest (e.g. microglia, astroglia) from regions of interest (e.g. cortex, hippocampus, thalamus); 2) by using RNA sequencing, which allows full sequencing of the entire transcriptome and, unlike microarrays, is sensitive to lowly expressed genes 3) by following up transcriptome-wide analyses with RT-qPCR and protein analyses.

Transcriptome-wide analyses could also be carried out in the piglet, however adequate sample size for genome-wide analyses is considerably more difficult to obtain than in rodents. Nonetheless, the piglet brain is more similar to the human brain in terms of anatomical complexities, myelination, genome as well as development, contrary to the rodent

brain which has a major postnatal growth spurt (1504, 1508-1511). The HI insult is also more similar since it is global rather than focal (e.g. hypoxia combined with airway occlusion causing cardiac arrest followed by resuscitation) and the neurological and neurobehavioural impairment resemble those seen in humans (1512).

In newborns with HIE, blood-based transcriptomic analyses have revealed widespread gene expression dysregulation compared to controls (1513). Major pathways include axonal development, adhesion and infiltration of peripheral granulocytes (e.g. neutrophils, mast cells), IL12 signalling and production in macrophages and HIF1α signalling. The overlap in gene expression differences with a subset of newborns with neonatal sepsis was relatively modest and effects largely had opposite direction. Thus, while both HIE and sepsis are associated with inflammation, the mechanisms of inflammation may be divergent.

Transcriptome-wide gene expression differences present in blood at birth are also associated with adverse outcomes at 18 months (Bayley scores, GMFCS scores, blindness, deafness) (1514). Major pathways include melatonin, which protects from free radicals, and polo-like kinase signalling, which triggers apoptosis following HI. Both of these pathways trigger HIF1α signalling, supporting the central role of this gene in HIE (see 5.2.3.2). Such transcriptomic analyses may provide a useful clinical tool for rapid stratification of HIE at birth and identification of candidate drug targets. Epigenomic analyses may complement such studies by examining epigenetic regulation affecting gene expression

5.2.3.2 Epigenomic studies

While no studies have assessed the effect of acute HI on global DNA methylation in HIE models, evidence is emerging from models of chronic hypoxia during pregnancy, either in isolation or in a multiple hit paradigm. These studies have provided evidence of global and long-lasting changes in DNA methylation and expression in the developing brain following chronic antenatal hypoxia, which sensitise the brain to a subsequent acute HI insult around term time (985, 1016, 1017). Such changes include, for example, upregulation of the *Wnt/β-catenin* signalling pathway, which plays a key role in brain development and synaptogenesis and is dysregulated in neurodevelopmental and neurological disorders (1515-1517). The glucocorticoid receptor, involved in the stress response, is also affected, with hypermethylation and gene silencing worsening injury induced by the second hit of HI (1518). This *in vivo* evidence is complemented by *in vitro* studies of hypoxia in rodent

hippocampal neurons, showing a tendency for promoters and CpG islands across the genome to remain hypomethylated long after the end of a mild and short hypoxic insult (1519).

Hypoxia is thought to impact epigenetic regulation via multiple mechanisms. Firstly, hypomethylation and upregulation of HIF1α seems to be central to the sensitisation process. HIF1α is the key mediator of the cellular response to hypoxia. It regulates many genes downstream, enabling adaptation to a hypoxic environment in the long-term by modulating angiogenesis, iron and glucose metabolism and stem cell function. However, it also affects expression of genes which may contribute to injury in the early phases and/or the long-term, including *Wnt* signalling and matrix metalloproteinases affecting the blood brain barrier (985, 1016). Some evidence exists of a bidirectional relationship with pro-inflammatory cytokines in normoxia, hypoxia and inflammation (1520-1525) and the participation of HIF1α in inflammatory processes is emerging (1526), however more data specific to the developing brain is required. Moreover, HIF1α also affects epigenetic regulators, including miRNAs and histone methylases, further propagating the epigenetic effects downstream (1017, 1527-1532). The complex roles of HIF1α are evidenced by the fact that early inhibition after HI is neuroprotective while later inhibition or genetic deletion exacerbates injury (1533-1535).

Evidence from adult stroke and cancer studies suggests that hypoxia also directly affects the "writers" (DNMTs) and "erasers" (TETs) of DNA methylation (1536-1539). For example, disruption of cell metabolism induced by hypoxia can reduce availability of substrates necessary for TET activity (e.g. α-ketoglutarate) and produce new metabolites that inhibit TET (1540, 1541). Reverse glutamate transport may exacerbate the reduced availability of α-ketoglutarate, since when glutamate is depleted it can be rapidly synthesised *de novo* from α-ketoglutarate (1540, 1542).

Finally, adult model studies show that HI can additionally alter DNA methylation without requiring enzyme activity. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can chemically alter cytosines directly, so that they mimic 5-mC and induce DNMTs improperly leading to gene silencing (1543). ROS and RNS can also promote processing of methylated cytosines to 5-hmC, which are not recognised by the "readers" of DNA methylation (e.g. MeCP2) and therefore fail to engage co-operative epigenetic mechanisms (1544, 1545).

Future work could assess global DNA methylation changes in this Rice Vannucci model alongside co-regulation of other epigenetic mechanisms. Of relevance, evidence is emerging that hypoxia and HI also alter histone marks and miRNAs (1530). In a preconditioning model assessing the mechanisms through which a mild and short hypoxic insult during pregnancy protects the brain from a more severe hypoxic insult at birth, upregulation of histone deacetylation seems to be central. Such upregulation, which promotes chromatin condensation and transcriptional repression, is indeed not seen without the preconditioning insult (1546, 1547). This suggests that the epigenetic and transcriptional profiles may be unique depending on the nature of the insult (hypoxia vs HI, acute vs chronic, global vs local), number of hits (single vs multiple) and severity. The nature of epigenetic changes following perinatal stresses is likely to depend not only on the type of insult but also on degree of brain maturity. For example, neuroinflammation in a mouse model of IL1β-induced preterm brain injury leaves chromatin accessibility unaffected in pre-oligodendrocytes, while taking advantage of chromatin regions already open during early development (1487).

Importantly, DNA methylation can now be manipulated in animal models, via optogenetic methods and the CRISPR-Cas9 technology (1548-1550). These methodologies will be helpful in studying the effects of DNA methylation changes *in vivo* once more robust evidence emerges for particular candidate genes. For example, the CRISPR-Cas9 technology could be used not only to edit the sequence of gene promoters and assess the effects of the regulatory SNPs on expression, but also activate or repress transcription by editing DNA methylation and histone acetylation status (1551).

In humans, no EWAS has yet assessed methylation in term newborns with HIE compared to term-born controls. With a prevalence of 2-3/1,000 newborns, reaching adequate sample size is a challenge. On the other hand, EWAS have so far pointed to widespread peripheral methylation differences in preterm vs term newborns, including genes involved in labour and delivery, brain development and immunity (1292, 1552-1559). Widespread peripheral methylation differences have also been found in sick preterm newborns vs healthy preterm newborns in the NICU, including at glutamate receptors in relation to cumulative scores for neurological morbidity (1560, 1561). Methylation differences have been shown to be present at birth in pairs of monozygotic twins (mostly preterm) who later became discordant for CP, including genes involved in responses to inflammation (e.g. TNFβ, NFκB) and hypoxia (e.g. HNRNPL) (1562). Another twin CP study measured DNA methylation in childhood rather than at birth, revealing differences in key biological processes, including the cytokine

signalling pathway, cell transport, growth and death, and genetic processes (1563). Importantly, measuring DNA methylation in childhood gives a snapshot of DNA methylation differences but provides no mechanistic insights into timing and causes of DNA methylation changes.

Compared to GWAS, EWAS are complicated by the fact that the epigenome is a phenotype, which varies with cell type, tissue, developmental stage, age, environmental exposures, genetic background and by chance (933, 1564, 1565). Access to the target tissue of disease, the brain, is generally not possible or difficult in humans. EWAS can be carried out in post-mortem brain tissue (e.g. HIE and cPVL brains), however this does not offer insights into causation since methylation is measured after disease manifestation and possibly treatment, and DNA degrades with increasing post-mortem interval (1013, 1566, 1567). So far, most human epigenetic studies of brain-based disorders have assessed DNA methylation in peripheral tissues, taken additional steps to assess relevance to the brain and discussed inherent limitations. Several studies have shown a good degree of correlation between brain and blood methylation, especially at CpG islands compared to other promoter regions and gene bodies (1568, 1569). Recent evidence suggests saliva may be a better surrogate tissue in EWAS of brain disorders, likely due to the variety and mixed proportions of cell types in saliva compared to blood and buccal swabs (1540, 1570-1572).

If the goal of the EWAS is to gain mechanistic insights, further steps need to be undertaken to strengthen causal inferences from peripheral methylation. This is achieved by choosing study designs and analytical approaches that account as much as possible for observational issues (reverse causation, confounding, selection bias) and biological confounders (e.g. differences in cell composition in the sample, genetic differences affecting DNA methylation, genetically determined differences in transcription affecting DNA methylation) (933, 1184). This may include longitudinal designs in prospective birth cohorts (e.g. ALSPAC) minimising issues of reverse causation and confounding due to choice of controls, and allowing assessment of DNA methylation stability (933). Mendelian randomisation approaches can also be used to strengthen causal inferences for EWAS. This relies on robust associations for SNP-DNA methylation (mQTL effects) and SNP-outcome associations from GWAS studies (1018, 1573). While mQTL data can be obtained from publicly available databases from GWAS of DNA methylation in foetal brain (http://epigenetics.essex.ac.uk/mQTL/) and adult brain (http://mostafavilab.stat.ubc.ca/xqtl), the main limiting factor for this type of analysis remains the lack of GWAS of newborn brain injuries and CP. The most comprehensive analytical

framework for assessment of DNA methylation as a causal mediator between perinatal exposures and brain injuries or CP would be a two-step MR analysis (1018). This approach has recently shown that DNA methylation is a mediator between prenatal vitamin B12 and cognitive abilities (1574). This type of analysis requires not only GWAS of relevant outcomes for the second step (effect of DNA methylation on outcome), but also GWAS of perinatal exposures for the first step (effect of the exposure on DNA methylation), which are also currently scarce. Relevant GWAS studies are therefore urgently needed, not only for investigation of the genetic architecture of brain injuries and CP, but also for understanding the role of epigenetic factors.

When the pathway to injury is thought to start from a methylation change in the brain which subsequently affects the periphery (or at the very least a common cause affecting both brain and periphery simultaneously), concordance between brain and blood methylation must also be considered before attempting causal interpretations (1019). Databases with matched pre-mortem blood methylation and post-mortem brain methylation have been made available to the scientific community to evaluate concordance of any regions of interest in studies of adult brain disorders (1019, 1575). Such matched databases do not yet exist for newborns. Animal model studies are particularly useful in this scenario.

An entirely separate goal of EWAS studies is that of prediction. In the context of biomarker studies, as long as DNA methylation is robustly associated with the exposure or the disease, it does not matter whether it is causally involved or it simply reflects biological confounders (e.g. cell type composition of the sample) (933, 1576). To this end, cord blood DNA methylation is currently being assessed for association with a range of early life stresses (e.g. maternal depression and anxiety, neurotoxin exposure) (1577). This approach may have great clinical utility for identifying peripheral biomarkers for HIE, and for improving speed and accuracy of CP diagnoses. This was shown by an EWAS of unrelated teenagers with CP, whereby observed DNA methylation differences were used to build a model which predicted CP diagnoses with relatively good accuracy in an independent sample of 4 year old children (1578).

5.2.3.3 Single cell multi-omics studies

One of the main challenges in epigenetic studies is that, unlike the genome, the epigenome varies in different cells and tissues, and both brain and blood are heterogeneous collections of different cell types. This has implications for animal model studies such as this one, where

analyses are carried out in bulk tissue and can only detect aggregate measures of gene expression or DNA methylation. In future studies, astrocytes or neurons could be isolated by FACS, before separating DNA and mRNA from single cells for parallel sequencing of the genome, epigenome, and transcriptome (1579, 1580). Integration of genomic, transcriptomic, epigenomic and electrophysiological information from single neurons and glial cells would allow a true understanding of gene regulation and how it underpins cell-specific behaviour in health, disease, and development (1550, 1581).

6 Conclusion

In this project, the role of genetic, transcriptional, and epigenetic regulation of astrocytic glutamate transport (*EAAT2*) and inflammation (*TNFα*, *IL1β*, *IL6*) was assessed in relation to newborn brain injuries (cPVL in preterm newborns and HIE in term newborns) and subsequent motor and cognitive impairment.

Genetic variants associated with higher cytokine levels, and therefore potentially stronger inflammatory responses, were found to be more frequent in children with cPVL and cerebral palsy (*TNFα* -308) and in association with cognitive scores (British Ability Scales) at 5 years of age (*IL1β* -511), especially the non-verbal reasoning subscale. There was also suggestive evidence for variants in *IL6* (-174) with moderate to severe white matter injury at birth, and variants in *EAAT2* (-200/-181) with verbal skills at 5 years of age.

In an established rat model of moderate hypoxic-ischemic brain injury at term, hypoxia-ischaemia induced expression of all three cytokines in both cortex and hippocampus in the first 24h, with a trend for early peak at 6h. This neuroinflammatory response was accompanied by changes in brain injury markers suggesting onset of astrogliosis and myelin injury. DNA methylation at the promoter of these cytokines could not be assessed within the timeframe of the project. However, it remains a suitable candidate mechanism for mediation of these transcriptional changes and holds potential for clinical use as a biomarker of HIE in newborns when measured in blood. Accumulating evidence of neuroinflammation following hypoxia-ischaemia supports stratification of HIE newborns based on neuroinflammatory status at birth and research into the neuroprotective effects of drugs modulating inflammation.

Evidence of a potential suppression of the glutamate transporter following hypoxia-ischaemia in the cortex was weak, and it was not associated with DNA methylation changes in any of

the three promoter regions examined. Inclusion of good quality DNA methylation data for imprinted gene *Peg3* strongly suggests that these findings are true negatives with these experimental conditions and sample size. More insights might be gained by purifying astrocytes, focusing on a later developmental stage, assessing more dynamic types of epigenetic mechanisms sensitive to the environment, as well as post-translational regulation.

7 References

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8 Appendix

Table 8.1. Literature review of candidate cytokine SNP-relevant outcome studies

Detrimental effects are highlighted in orange, protective effects in purple, no effects are not highlighted

Genetic Variant	Study Design and Sample Size	Demographics	CP (n) in subset analysis	Gestational Age	Outcome	Finding	Reference
TNFα -308 G>A	443 cases, 883 controls	White Australian	27	<32	CP (HP)	AA+GA vs GG (OR=2.38 (1.02-5.58))	(906)
			65	≥37	CP (QP)	GA vs GG (OR=1.82 (1.04-3.15))	
	587 cases, 1154 controls		141	All	CP (QP)	A (OR=0.62 (0.43-0.89)	(917)
			82	32-36	CP	AA vs GA+GG (OR=0.31 (0.14-0.69))	(162)
			648	≥36	CP	A (OR=0.79 (0.63-1.00)	
			282	All	CP (QP)	A (OR=0.63 (0.44-0.90)	
	60 cases, 84 controls	White Croatian	60	<32	CP	GA vs GG (OR=0.39 (0.17-0.90))	(908)
	96 cases, 119 controls	Mixed North Americans	96	<32	CP	AA vs GG (OR=0.43 (0.02-10.60); GA vs	(910)

						GG (OR=1.18 (0.63-2.23))	
	138 cases, 165 controls	White North American	138	≥37	CP	AA vs GG (OR=1.9 (0.04-8.0); GA vs GG (OR=0.8 (0.5-1.3))	(1115)
	125 cases, 147 controls	Mixed North Americans	100	All	Bayley<85 in the absence of CP	No association (OR not reported, manually calculated: AA vs GG OR= 0.32 (0.01-6.71); GA vs GG OR=1.22 (0.61-2.42))	(905)
	211 cases, 195 controls	Mixed North Americans	Not reported	<32	Stillbirth/death within 1 y OR CP or Bayley<70	No association (OR not reported)	(919)
Il1β -511 C>T	587 cases, 1154 controls	White Australian	587	All	CP	No association (OR not reported)	(917)
			132	All	Other CP types	T (OR=0.56 (0.37-0.86)	(162)
	60 cases, 84 controls	White Croatian	60	<32	CP	TT vs CC (OR=1.85 (0.71-4.82); CT vs CC (OR=1.76 (0.84-3.70))	(908)

			35	<32	CP + cPVL	TT vs CC (OR=3.22 (1.11-9.31)); T vs CC (OR=3.22 (1.11-9.31)); T (OR=1.96 (1.11-3.44))	
	125 cases, 147 controls	Mixed North Americans	100	All	Bayley<85 in the absence of CP	OR not reported, manually calculated: TT vs CC (OR= 3.58 (1.36-9.39)); CT vs CC (OR=1.44 (0.75-2.76))); TT vs CT+CC (OR=3.1 (1.2-8.2))	(905)
	211 cases, 195 controls	Mixed North Americans	Not reported	<32	Stillbirth/death within 1 y OR CP or Bayley<70	No association (OR not reported)	(919)
	50 cases, 50 controls	Mixed Brasilian	50	All	cPVL + Neonatal encephalopathy	CT + TT vs CC (OR=23.12 (1.31-409.4))	(1028)
IL6 -174 C>G	60 cases, 84 controls	White Croatian	60	<32	CP	GG vs CC (OR=0.84 (0.31-2.28)); GC vs CC (OR=1.05 (0.43-2.58))	(908)

	125 cases, 147 controls	Mixed North Americans	100	All	Bayley<85 in the absence of CP	OR not reported, manually calculated: CC vs GG (OR= 2.39 (0.45-12.56)); CG vs GG (OR=0.89 (0.16-5.06)); C vs G (OR=2.12 (1.11-4.02))	(905)
	211 cases, 195 controls	Mixed North Americans	Not reported	<32	Stillbirth/death within 1 y OR CP or Bayley<70	No association (OR not reported)	(919)
	Kaiser Permanente Medical Care Program cohort (n=334,333)	Mixed North Americans	250 (model adjusted for race)	≥36	CP	CC vs GG (OR= 2.6 (1.5-4.7)); C (OR= 1.5 (1.2-2.0))	(163)
			65 (model adjusted for race)		CP (QP)	CC vs GG (OR=4.1 (1.8-9.3))	
			97 (model adjusted for race)		CP (HP)	CC vs GG (OR=2.7 (1.3-5.7))	
			63 (model adjusted for race)		CP (DP)	CC vs GG (OR=1.7 (0.7-4.1)	
	250 cases, 305 controls	Mixed North Americans	250	≥36	CP	CC vs GG (OR=2.5 (1.4-4.6))	(918)

	587 cases, 1154 controls	White Australian	587	All	CP	No association (OR not reported)	(917)
						CC+CG vs GG) (OR=1.35 (1.04-1.75))	(162)
			324	≥36	CP	CC+CG vs GG) (OR=1.40 (1.02-1.92))	
			189	All	CP (HP)	CC+CG vs GG) (OR=1.55 (1.06-2.27))	
	443 cases, 883 controls	White Australian	67	All	CP (HP)	CC vs GG (OR=1.89 (1.12–3.18))	(1027)
			17	32-26	CP (QP)	CC + CG vs GG (OR=10.42 (1.34–80.82))	
	52 cases, 46 controls	White Austrian	52	<36	cPVL + mental retardation	CC vs GG (RR=3.11 (1.54-6.29)); CG vs GG (RR=1.79 (1.10-2.92)). No Association with cPVL or CP	(1032)
	713 cases, 753 controls	Han Chinese	56	All	CP + cPVL in males	OR not reported, manually calculated for CC + CG vs GG	(654)

						(OR=1.92 (1.10–3.37)) No association with CP	
	APIP cohort (n=308)	White British	148	≤32	cPVL	CC vs CG + GG (OR=4.1 (1.4–12.2))	(1030)
					Severe PVH	CC vs CG + GG (OR=3.5 (1.0–12.2))	
					Any disability (motor, vision, hearing, and cognitive)	CC vs CG + GG (OR=2.8 (1.04–7.4)). No association with CP	
	Cohort (n=1202)	79% German, 90% European	1202	>24 and ≤37 + <1,500 g	IVH grade IV, cPVL, ventricular-peritoneal-shunting or death	No association (CC: 12%, CG: 9%, GG: 8%)	(1029)
	PIPARI cohort (n=175)	Finnish	153	<32 or <1,500g	Reduced volume of deep grey matter (basal ganglia + thalamus)	CC vs GG (difference in means = -3.19, -5.61--0.78) No association with ultrasound abnormalities (cPVL, IVH).	(1031)
EAAT2 -181 A>C and -200 C>A	APIP cohort (n=541)	92% White British	314 (model adjusted for sex, birth weight,	≤32	CP	-200/181 Genotype Combination: A at -200 (OR=	(911)

			gestational age and Apgar scores at 1 and 5 min)			4.34 (1.12–16.77) + A at -181 (OR= 6.64 (1.76–25.07))	
					Low Neurodevelopmental Score: Lowest 10th centile for Griffith or Bayley Scores	-200/181 Genotype Combination: A at -181 (OR= 4.15 (1.05–16.38))	

Table 8.2. Regression modelling of the EAAT2-TNFα SNP effects on cerebral palsy

Logistic Regression Model for Cerebral Palsy	**Genetic Exposures**	**Likelihood ratio test**	**Unadjusted Analyses** (n=178)	
		p	**OR (95% CI)**	**p**
Univariable	*EAAT2 (No. A alleles) -200/-181*	0.02 (vs baseline)	7.65 (1.40-41.68)	0.02
	TNFα -308	0.07 (vs baseline)		
	GA		2.09 (0.69-6.32)	0.19
	AA		6.71 (1.38-32.74)	0.02
Multivariable (Main Effects)	*EAAT2 (No. A alleles) -200/-181*	0.06 (vs univariable - EAAT2)	9.22 (1.55-54.78)	0.02
	TNFα -308			
	GA	0.02 (vs univariable - TNFα)	2.21 (0.70-6.91)	0.18
	AA		7.35 (1.47-16.76)	0.02
Multivariable (Interaction)	*EAAT2 (No. A alleles) -200/-181*TNFα(GA)*	0.04 (vs multivariable - main effects)	9.47e+07 (0)	1.00
	*EAAT2 (No. A alleles) -200/-181*TNFα(AA)*		Not estimable (no children with *TNFα(AA)* and 1 or 3 A alleles at EAAT2 -200/-181)	-

Table 8.3. Regression modelling of the EAAT2-TNFα SNP effects on the BAS cognitive score at 5 years

Linear Regression Model for the BAS Score	**Genetic Exposures**	**Likelihood ratio test**	**Unadjusted Analysis** (n=114)	
		p	**β (95% CI)**	**p**
Univariable	*EAAT2 (No. A alleles) -200/-181*	0.08 (vs baseline)	-7.06 (-15.17-1.06)	0.09
	TNFα -308	0.18 (vs baseline)		
	GA		-4.12 (-10.01-1.77)	0.17
	AA		5.93 (-7.02-18.88)	0.37
Multivariable (Main Effects)	*EAAT2 (No. A alleles) -200/-181*	0.16 (vs univariable - EAAT2)	-7.27 (-15.33-0.79)	0.08
	TNFα -308			
	GA	0.07 (vs univariable - TNFα)	-4.20 (-10.04-1.64)	0.16
	AA		6.16 (-6.66-18.99)	0.34
Multivariable (Interaction)	*EAAT2 (No. A alleles) -200/-181*TNFα(GA)*	0.01 (vs multivariable - main effects)	-20.50 (-36.40--4.60)	0.01
	*EAAT2 (No. A alleles) -200/-181*TNFα(AA)*		Not estimable (no children with *TNFα(AA)* and 1 or 3 A alleles at EAAT2 -200/-181)	-

Figure 8.1. Plots of model predictions of the effect of the increasing number of A alleles at EAAT2 -200/-181 on the BAS score by TNFα -308 risk genotypes

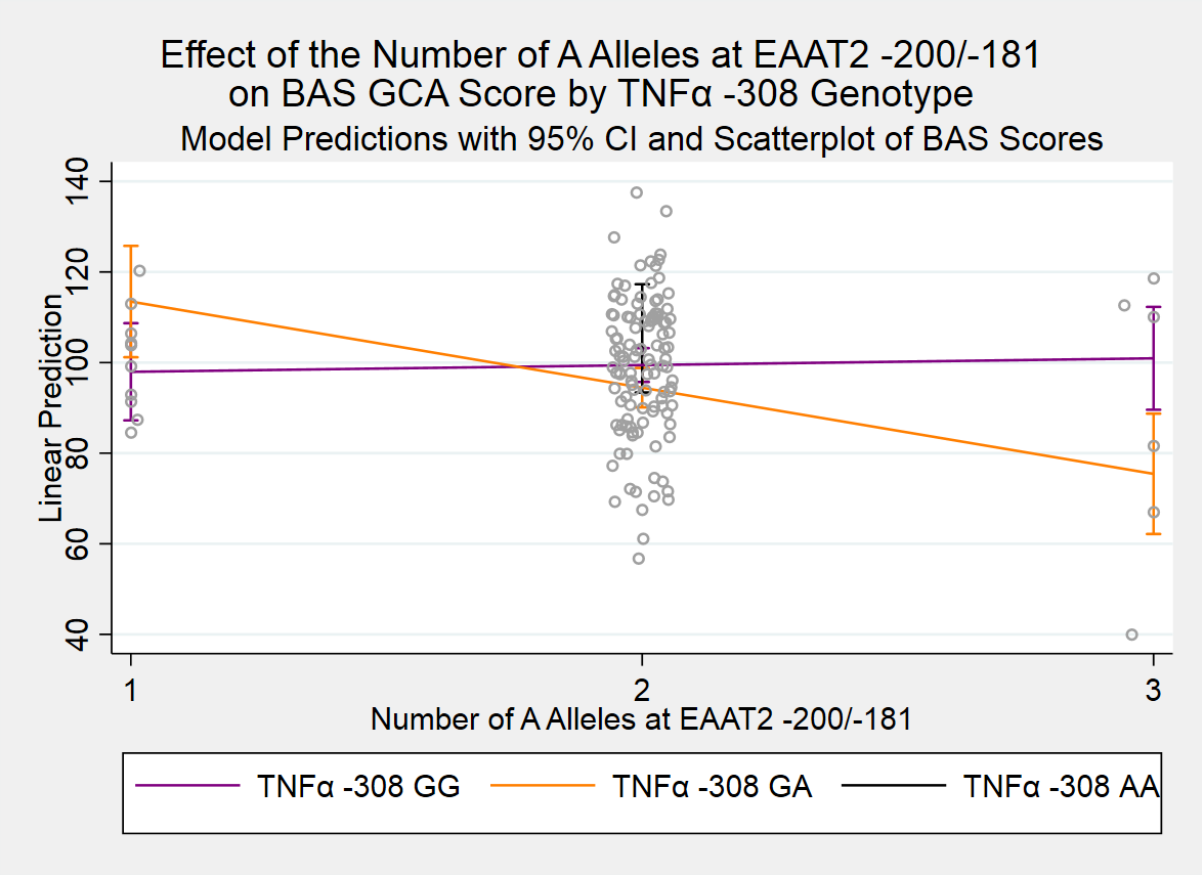

Table 8.4. Generalised linear model of the EAAT2-TNFα SNP effects on the M-ABC motor score at 5 years

Generalised Linear Model for the M-ABC Score (gamma family, log link)	**Genetic Exposures**	**Likelihood ratio test**	**Unadjusted Analysis** (n=121)	
		p	**β (95% CI)**	**p**
Univariable	*EAAT2 (No. A alleles) -200/-181*	0.17 (vs baseline)	0.33 (-0.17-0.84)	0.20
	TNFα -308	0.52 (vs baseline)		
	GA		-0.02 (-0.43-0.38)	0.92
	AA		0.41 (-0.43-1.25)	0.34
Multivariable (Main Effects)	*EAAT2 (No. A alleles) -200/-181*	0.53 (vs univariable - EAAT2)	0.32 (-0.18-0.82)	0.21
	TNFα -308			
	GA	0.18 (vs univariable - TNFα)	-0.04 (-0.44-0.36)	0.84
	AA		0.39 (-0.45-1.22)	0.37
Multivariable (Interaction)	*EAAT2 (No. A alleles) -200/-181*TNFα(GA)*	0.59 (vs multivariable - main effects)	0.26 (-0.76-1.28)	0.62
	*EAAT2 (No. A alleles) -200/-181*TNFα(AA)*		Not estimable (no children with *TNFα(AA)* and 1 or 3 A alleles at EAAT2 -200/-181)	-

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Glutamate Transport and Preterm Brain Injury

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Preterm birth complications are the leading cause of child death worldwide and a top global health priority. Among the survivors, the risk of life-long disabilities is high, including cerebral palsy and impairment of movement, cognition, and behavior. Understanding the molecular mechanisms of preterm brain injuries is at the core of future healthcare improvements. Glutamate excitotoxicity is a key mechanism in preterm brain injury, whereby the accumulation of extracellular glutamate damages the delicate immature oligodendrocytes and neurons, leading to the typical patterns of injury seen in the periventricular white matter. Glutamate excitotoxicity is thought to be induced by an interaction between environmental triggers of injury in the perinatal period, particularly cerebral hypoxia-ischemia and infection/inflammation, and developmental and genetic vulnerabilities. To avoid extracellular build-up of glutamate, the brain relies on rapid uptake by sodium-dependent glutamate transporters. Astrocytic excitatory amino acid transporter 2 (EAAT2) is responsible for up to 95% of glutamate clearance, and several lines of evidence suggest that it is essential for brain functioning. While in the adult EAAT2 is predominantly expressed by astrocytes, EAAT2 is transiently upregulated in the immature oligodendrocytes and selected neuronal populations during mid-late gestation, at the peak time for preterm brain injury. This developmental upregulation may interact with perinatal hypoxia-ischemia and infection/inflammation and contribute to the selective vulnerability of the immature oligodendrocytes and neurons in the preterm brain. Disruption of EAAT2 may involve not only altered expression but also impaired function with reversal of transport direction. Importantly, elevated EAAT2 levels have been found in the reactive astrocytes and macrophages of human infant post-mortem brains with severe white matter injury (cystic periventricular leukomalacia), potentially suggesting an adaptive mechanism against excitotoxicity. Interestingly, EAAT2 is suppressed in animal models of acute hypoxic-ischemic brain injury at term, pointing to an important and complex role in newborn brain injuries. Enhancement of EAAT2 expression and transport function is gathering attention as a potential therapeutic approach for a variety of adult disorders and awaits exploration in the context of the preterm brain injuries.

Keywords: preterm infant, brain injury, glutamate, excitotoxicity, inflammation, EAAT2, SLC1A2, GLT-1

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GLOBAL SIGNIFICANCE OF PRETERM BRAIN INJURIES

Perinatal care has advanced considerably in the last century and has improved survival of many vulnerable newborns, including those born preterm. The World Health Organization estimates that 15 million newborns (1 in 10 live births) are born preterm (<37 weeks of gestation) worldwide each year (World Health Organization, 2012). Despite global improvements, the United Nations Millennium Development Goal to reduce childhood mortality by two-thirds in 2015 was not achieved globally (United Nations, 2015) and 2.7 million children died in the first month of life worldwide in 2015. Of these babies, over 900,000 died due to preterm birth complications – the leading cause of death of newborns and children under 5 years old (Liu et al., 2016). For the newborns who survive, the multi-organ damage can result in life-long disabilities. Globally, preterm birth complications represent the fourth leading cause of years of "healthy" life lost due to disability (i.e., over 102,000 DALYs), above causes such as diarrheal diseases, diabetes, and HIV (World Health Organization, 2016).

Prematurity is a major risk factor for cerebral palsy, "a group of permanent disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain" (Bax et al., 2005; Rosenbaum et al., 2007). Cerebral palsy is the most common physical disability in childhood and is a heterogeneous diagnosis, including different clinical types and brain imaging patterns, comorbidities, and multiple causes (Stanley et al., 2000; Locatelli et al., 2010; MacLennan et al., 2015). Preterm birth is clearly an important risk factor and risk is 30 times higher in children born before 33 weeks of gestation than in those born at term (Stanley, 1992; Himpens et al., 2008; Beaino et al., 2010; Mercier et al., 2010; Tronnes et al., 2014; MacLennan et al., 2015; Stavsky et al., 2017). A recent meta-analysis estimated an increase in prevalence from 1.4/1,000 live births in children born at term (>36 weeks of gestation) to 6.8/1,000 live births in moderate to late preterm (32–36 weeks of gestation), rising to 43.2/1,000 live births in very preterm (28–31 weeks of gestation) and 82.3/1,000 live births in extremely preterm infants (<28 weeks of gestation) (Oskoui et al., 2013; Hirvonen et al., 2014). More than a third of the extremely preterm children with cerebral palsy are unable to walk (Moore et al., 2012), and many have multiple disabilities, which may further limit independence and quality of life (Litt et al., 2005; Glass et al., 2008, 2015; Soria-Pastor et al., 2008; Anderson et al., 2011; Moore et al., 2012). A systematic review of international cerebral palsy registers in high-income settings highlighted the extent of these comorbidities: around three quarters of children with cerebral palsy suffer from chronic pain; approximately half have intellectual disabilities (IQ, executive function, language ability); around a quarter have active epilepsy, hip dislocation, bladder control problems, behavioral problems, sleep disorders, and/or speech impairment; 11 and 4% have severe vision and hearing impairment, respectively (Novak et al., 2012). There are less data from low-income settings, but it is likely that comorbidities, as well as mortality, are higher (Khandaker et al., 2015). Preterm birth complications impose a considerable economic burden on the public sector, which was estimated around £2.9 billion in England and Wales in 2006 (Mangham et al., 2009). While administration of magnesium sulfate as a preventative treatment to the mother during preterm labor has been shown to reduce risk of cerebral palsy by a third in very preterm infants (Doyle et al., 2009), no postnatal therapy currently exists for preterm brain injury. This is a global health priority as the increase in both preterm birth and survival rates has not been matched by a decrease in long-term disability (Wilson-Costello et al., 2005).

NEUROIMAGING AND NEUROPATHOLOGY OF PRETERM BRAIN INJURIES

Preterm birth is associated with smaller brain volumes (Peterson et al., 2003; Inder et al., 2005; Srinivasan et al., 2007) as well as motor, cognitive, and behavioral problems at school age (Peterson et al., 2000, 2003; Abernethy et al., 2004; Nosarti et al., 2005; Gimenez et al., 2006; Anderson and Doyle, 2008; Kesler et al., 2008; Aarnoudse-Moens et al., 2009; Delobel-Ayoub et al., 2009; Soria-Pastor et al., 2009; Anderson et al., 2017). Progress in neuroimaging techniques has been key in linking childhood neurodevelopmental outcomes to perinatal brain injuries and in advancing our knowledge of the underlying neuropathology (Volpe, 2009c; Back, 2017). Both MRI-defined preterm white matter injury (periventricular leukomalacia) and preterm birth are predictive of cerebral palsy (Constantinou et al., 2007; Spittle et al., 2008, 2009, 2018; Duerden et al., 2013). In a large European population study of cerebral palsy, white matter injury was the most common feature found in over 40% of the children (Bax et al., 2006). Originally, cranial ultrasound could only detect the most severe cystic type of white matter injury (cystic periventricular leukomalacia), characterized by focal macroscopic cysts of necrotic tissue in the deep white matter (de Vries et al., 1992) and highly predictive of cerebral palsy (Leviton and Paneth, 1990; De Vries et al., 2004; Serdaroglu et al., 2004; Fetters and Huang, 2007). Necrotic white matter injury can also evolve into microscopic glial scars, which may not be visible with traditional ultrasound. These are a more common type of injury and are sufficient to cause a loss in brain volume (Volpe, 2009c; Volpe et al., 2011). With the development of MRI techniques, a diffuse type of white matter injury has increasingly been recognized in the form of diffuse disturbances of myelination in the central white matter. This has emerged as the predominant type of white matter injury, accounting for over 90% of periventricular leukomalacia cases, as well as the predominant type of preterm brain injury altogether, occurring in 50% preterm newborns (Volpe, 2008). Importantly, while rates of the more severe cystic form have declined to less than 5% with advances in perinatal care, this has not been reflected for the diffuse forms (Maalouf et al., 2001; Counsell et al.,

2003; Inder et al., 2003; Miller et al., 2003; Back et al., 2007b; Volpe, 2008). These could be seen as different manifestations of an "encephalopathy of prematurity" (Volpe, 2009c) or even as distinct pathologies (Back and Rosenberg, 2014). In the last two decades, advanced MRI techniques have highlighted that injury is not limited to the white matter but it extends to the deep grey matter, cortex, and cerebellum, all of which contribute to the volume loss (Counsell and Boardman, 2005; Ball et al., 2012). The cerebellum is gathering attention as a key target of injury. This region grows rapidly at the peak time for preterm birth and damage in the form of infarction, atrophy, and poor growth has been reported as common in very preterm infants developing cerebral palsy and long-term motor, cognitive, and behavioral impairment (Mercuri et al., 1997; Abraham et al., 2001; Bodensteiner and Johnsen, 2005; Johnsen et al., 2005; Limperopoulos et al., 2005a,b, 2007; Nosarti et al., 2008; Parker et al., 2008; Lawrence et al., 2014). Indeed, there is a relationship between cerebellar volume loss and white matter injury, pointing to the existence of a common insult, such as hypoxia-ischemia and infection/inflammation, which are known to damage the developing cerebellum (Shah et al., 2006; Volpe, 2009b; Hutton et al., 2014).

Disentangling the spatial and temporal contributions of infection/inflammation and hypoxia-ischemia will be key in understanding brain injuries across the perinatal spectrum. For example, while white matter injury is typical of the preterm newborn, it may be present in a subset of newborns born at term who experienced *in utero* hypoxic-ischemic insults (e.g., placental insufficiencies) (Mallard et al., 1998; Rees et al., 1998; Zhu et al., 2016). Indeed, newborns born at term with hypoxic-ischemic encephalopathy are also at high risk and up to 40% develop cerebral palsy (Gluckman et al., 2005; Shankaran et al., 2005; Azzopardi et al., 2009; Simbruner et al., 2010; Jacobs et al., 2011). Investigating the molecular basis for divergence between term and preterm injuries is paramount for development of age-appropriate pharmacological therapies.

PATHOGENESIS OF PRETERM BRAIN INJURIES

Brain injury is thought to be more common in preterm than term newborns for several reasons, including developmental and genetic vulnerabilities and differential exposure to adverse perinatal environments. A considerable body of *in vitro* and *in vivo* evidence points two potential triggers of injury, hypoxia-ischemia, and infection/inflammation (Volpe, 2008, 2009a; Deng, 2010; Volpe et al., 2011; Back and Rosenberg, 2014; Back, 2017). These insults are thought to interact in the vulnerable immature brain and converge onto three downstream mechanisms of injury: inflammation, glutamate excitotoxicity, and ultimately free radical attack, which directly damages cell components as well as triggering delayed cell death by apoptosis. Severity and temporal profile of hypoxia-ischemia and infection/inflammation, degree of brain maturity, comorbidities, sex, and genetic background may all contribute to individual differences in pathogenesis, clinical presentation, and individual susceptibility to injury. We will review the role of developmental vulnerabilities, infection/inflammation, and hypoxia-ischemia and bring the focus on the common downstream mechanism of glutamate excitotoxicity. We will then review the evidence linking glutamate transport to excitotoxic preterm brain injuries and highlight the current evidence supporting excitatory amino acid transporter 2 (EAAT2) as a potential therapeutic target.

Developmental Vulnerability

The brain undergoes rapid and critical developmental events during the peak time of premature brain injury (24–32 weeks), including neuronal migration, growth of axons and dendrites, synaptogenesis, development of the vascular system, and myelination. Interference with these natural trajectories determines selective cellular and regional vulnerabilities and may redirect subsequent development. Among their functions, oligodendrocytes are responsible for laying the highly specialized myelin membrane around axons and are therefore key for the development of the white matter. Myelination begins before birth and peaks in the first 2 years of postnatal life, with the intracortical fibers of the cortex being myelinated in the third decade. The process of myelination requires that oligodendrocytes first proliferate and develop into mature oligodendrocytes and then depose myelin around axons (Volpe, 2008). Around the peak time of preterm brain injury (28–32 weeks of gestation), the pre-oligodendrocyte stage still represents the majority of the oligodendrial pool in the very preterm brain (Iida et al., 1995; Back et al., 2001). Pre-oligodendrocytes are more vulnerable than mature oligodendrocytes to hypoxia-ischemia, infection/inflammation, oxidative damage, and ultimately cell death (Back et al., 1998, 2002, 2005, 2007b; Fern and Moller, 2000; Baud et al., 2004; Fragoso et al., 2004; Segovia et al., 2008; Volpe et al., 2011). Indeed, a unique feature of periventricular white matter injury is an arrest in the development of oligodendrocytes at the pre-oligodendrocyte stage, leading to the abnormal myelination patterns typically seen through MRI (Back et al., 2007b; Volpe et al., 2011). More severe necrotic injury extends to all the cell components, leading to cysts and exacerbating myelin injury via focal axonal degeneration (Laptook, 2016; Back, 2017). Concurrent developmental vulnerabilities include the limited ability of the immature brain to synthesize appropriate amounts of growth factors needed for brain development and self-protection, and an immature immune system, potentially promoting an excessive and sustained inflammatory response (Gilles et al., 2018).

Environmental Triggers of Injury: Hypoxia/Ischemia and Infection/Inflammation

Alongside the intrinsic developmental vulnerability of the immature brain, the preterm newborn is exposed to a range of potentially harmful exposures in the perinatal period. Supported by mounting experimental and epidemiological evidence, perinatal infection/inflammation leading to an overly

intense inflammatory response, or a "cytokine storm", has increasingly been recognized as a major risk factor not only for preterm birth but also for preterm white matter injury and long-term neurodisabilities (Yoon et al., 1996, 1997, 2000; Baud et al., 1999; Duggan et al., 2001; Dollner et al., 2002; Heep et al., 2003; Kaukola et al., 2004, 2006; Ellison et al., 2005; Bi et al., 2014). The preterm brain is often exposed to inflammation early during fetal development (e.g., maternal infections and chorioamnionitis) and usually for prolonged periods during postnatal life in the neonatal intensive care environment (e.g., neonatal infections, inflammatory comorbidities such as necrotizing enterocolitis), during critical phases of myelination and brain plasticity (Murphy et al., 1995; Grether and Nelson, 1997; Verma et al., 1997; Alexander et al., 1998; Dammann and Leviton, 1998, 2000, 2004; O'Shea et al., 1998; Leviton et al., 1999; Wu and Colford, 2000; Dammann et al., 2002; Rezaie and Dean, 2002; Stoll et al., 2002; Wu, 2002; Schlapbach et al., 2011; Hagberg et al., 2015; Anblagan et al., 2016). A combination of multiple inflammatory hits, antenatally and postnatally, has been shown to increase risk of brain injury and disability compared to single hits (Korzeniewski et al., 2014; van der Burg et al., 2016; Yanni et al., 2017). Indeed, pharmacological interventions targeting inflammation may have translational potential based on preclinical studies (Hagberg et al., 2015).

The role of hypoxia-ischemia in preterm brain injury is more controversial. In term newborns with hypoxic-ischemic encephalopathy, defined and acute hypoxic-ischemic events before or during birth (e.g., placental abruption, cord occlusion, and uterine rupture) are usually recognized by the clinician and represent the first step of a diagnosis of hypoxic-ischemic encephalopathy, aided by objective clinical and neuroimaging criteria. In the preterm newborn, a sentinel event is rarely recognized, and hypoxia-ischemia is generally assumed to have a more complex temporal profile, with intermittent or chronic nature (Laptook, 2016; Ohshima et al., 2016). However, it remains challenging to determine the individual contribution of hypoxia-ischemia among several coexistent factors, such as infection/inflammation, growth restriction, or hyperoxia (Gopagondanahalli et al., 2016). Physiologically, it is conceivable that the preterm brain is vulnerable to hypoxia-ischemia due to the anatomical and functional immaturity of the periventricular vasculature, which would make the periventricular white matter vulnerable to minor drops in cerebral perfusion (Takashima and Tanaka, 1978; Lou et al., 1979; De Reuck, 1984; Altman et al., 1988; Pryds, 1991; Miyawaki et al., 1998; Inage et al., 2000; Volpe, 2008; Laptook, 2016). The periventricular white matter has lower basal blood flow compared to grey matter regions in both humans (Greisen, 1986; Pryds et al., 1990) and the preterm fetal sheep (Szymonowicz et al., 1988; Gleason et al., 1989; Riddle et al., 2006). Further drops in blood flow are common in sick premature infants with respiratory disease due to lung immaturity (Soul et al., 2007). Mechanical ventilation may contribute to ischemia due to the vasoconstrictive effect of the induced cumulative hypocarbia (Shankaran et al., 2006). Perinatal hypoxic-ischemic episodes are also likely to play a key role, including ongoing placental pathologies, an overlapping risk factor for intrauterine growth restriction, low birthweight, and preterm birth. A meta-analysis recently reported an association between preterm brain injury and perinatal risk factors related to hypoxia-ischemia, including oligohydramnios, acidemia, low Apgar scores, apnea, respiratory distress syndrome, and seizures (Huang et al., 2017). However, the link between regional differences in blood flow and vulnerability to severe white matter injury is not consistent, and even in moderate ischemia, some regions of white matter are spared. This suggests that ischemia is necessary but not sufficient in isolation (Riddle et al., 2006; McClure et al., 2008; Back, 2017). Indeed, it has been suggested that more consistent evidence is needed to ascertain the specific role of hypoxic and ischemic events in preterm brain injury altogether and that future research should take into account contributions and interactions with other biological processes, including infection/inflammation and developmental vulnerability (Gilles et al., 2018). Importantly, the impact of hypoxia-ischemia on the cerebellum is also emerging, as shown by reports of volume loss and death of Purkinje cells and Bergmann glia in term newborns with hypoxic-ischemic encephalopathy and mid-late gestation fetal sheep exposed to asphyxia (Rees et al., 1997; Inage et al., 1998; Castillo-Melendez et al., 2004; Biran et al., 2012; Hutton et al., 2014). In an established mouse model of chronic hypoxia recapitulating perinatal brain injuries, damage to the cerebellum was reported in terms of a significant loss of GABAergic interneurons and a delay in dendritic arborization of Purkinje cells, followed by motor impairment and cerebellar learning deficits (Chahboune et al., 2009; Zonouzi et al., 2015; Sathyanesan et al., 2018).

Several experimental studies have shown that hypoxia-ischemia and infection/inflammation lead to worse brain and behavioral outcomes when they interact, and insults that are individually insufficient to cause injury can lead to injury when combined (Dommergues et al., 2000; Eklind et al., 2001; Lehnardt et al., 2003; Ikeda et al., 2004; Larouche et al., 2005; Favrais et al., 2007; Wang et al., 2007, 2009, 2010; Aden et al., 2010; van Tilborg et al., 2018). This has led to the multiple hit hypothesis of preterm brain injury, whereby a mild first event sensitizes the brain to subsequent insults (Leviton et al., 2013; Van Steenwinckel et al., 2014; Barnett et al., 2018). The current hypothesis is that hypoxia-ischemia triggers an inflammatory response *per se*. This additional endogenous response combined with the inflammation triggered by infection leads to a pro-inflammatory "cytokine storm," which is not matched by upregulation of anti-inflammatory cytokines and neurotrophic factors. This in turn sensitizes the brain to hypoxic-ischemic injury by enhancing glutamate excitotoxicity and damaging the blood-brain barrier (Hagberg et al., 2015). Tertiary mechanisms of injury, mediated by epigenetic modifications, may sustain the sensitization in the long term and interfere with remodeling and repair mechanisms (Dammann, 2007; Fleiss and Gressens, 2012).

A substantial body of experimental evidence suggests that glutamate excitotoxicity triggered by hypoxia-ischemia and/or

infection/inflammation plays a key role in the pathogenesis of preterm white matter injury (Hagberg et al., 2002; Johnston, 2005; Volpe, 2008; Deng, 2010; Volpe et al., 2011).

GLUTAMATE EXCITOTOXICITY IN THE PRETERM BRAIN

Glutamate Homeostasis and Dysregulation

Glutamate is the main excitatory neurotransmitter in the mammalian brain (Meldrum, 2000). It is essential for brain function, orchestrating not only fast excitatory neurotransmission but also long-lasting neuronal changes necessary for memory, learning, and cognition. It is also fundamental during brain development, due to its role in regulating formation and elimination of synapses, as well as neuronal migration, proliferation, and viability. Glutamate is abundant inside the brain cells, and most neurons and glial cells have glutamate receptors distributed across most cellular elements, highlighting the importance of glutamatergic systems for normal function (Curtis and Johnston, 1974; Watkins and Evans, 1981; Bliss and Collingridge, 1993; Newcomer et al., 2000; Platt, 2007). Stimulation of a glutamatergic neuron results in Ca^{2+}-dependent release of glutamate in the synapse by vesicular exocytosis. Extracellular glutamate binds to and activates post-synaptic ionotropic (NMDA, AMPA, and kainate receptors) and metabotropic (mGluR) glutamate receptors, stimulating the post-synaptic neurons *via* Ca^{2+} or Na^{+} influx and inducing intracellular signaling cascades that lead to physiological cellular responses, such as regulation of transcription factors and DNA replication (Nicholls and Attwell, 1990; Danbolt, 2001).

Glutamatergic transmission is terminated when glutamate transporters, expressed predominantly by astrocytes, slowly take up glutamate from the synaptic space (30 glutamate molecules per second at Vmax) (Otis and Kavanaugh, 2000; Bergles et al., 2002; Grewer and Rauen, 2005; Takahashi et al., 2015). In the preterm brain, glutamate transporters are also expressed by immature neurons and oligodendrocytes, although their significance is controversial, as reviewed below. In astrocytes, glutamate is converted to glutamine *via* glutamine synthetase. Glutamine is shuttled back into the pre-synaptic neuron, where it is converted into glutamate *via* glutaminase (**Figure 1**). The glutamate-glutamine cycle is not essential for supplying glutamate for neuronal release but is needed for normal glutamatergic transmission (Danbolt, 2001; Takahashi et al., 2015; Danbolt et al., 2016).

The ubiquity of glutamate is a double-edged sword: when homeostasis is disrupted, glutamate can turn into a potent neurotoxin. If the concentration of glutamate in the extracellular space rises above physiological levels, post-synaptic glutamate receptors are overactivated. This excessive activation, or excitotoxicity, leads to cell death *via* activation of suicide cell programs (apoptosis) (Danbolt, 2001; Sattler and Tymianski, 2001) (**Figure 1**). Since it was first proposed in the late 1960s (Olney, 1969), the concept of glutamate excitotoxicity has been implicated in several adult disorders, both acute (e.g., ischemic stroke and traumatic brain injury) and chronic (e.g., amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, major depression, and addiction) (Doble, 1999; Takahashi et al., 2015). Consistently, injection of glutamate agonists into the cortex, striatum, and

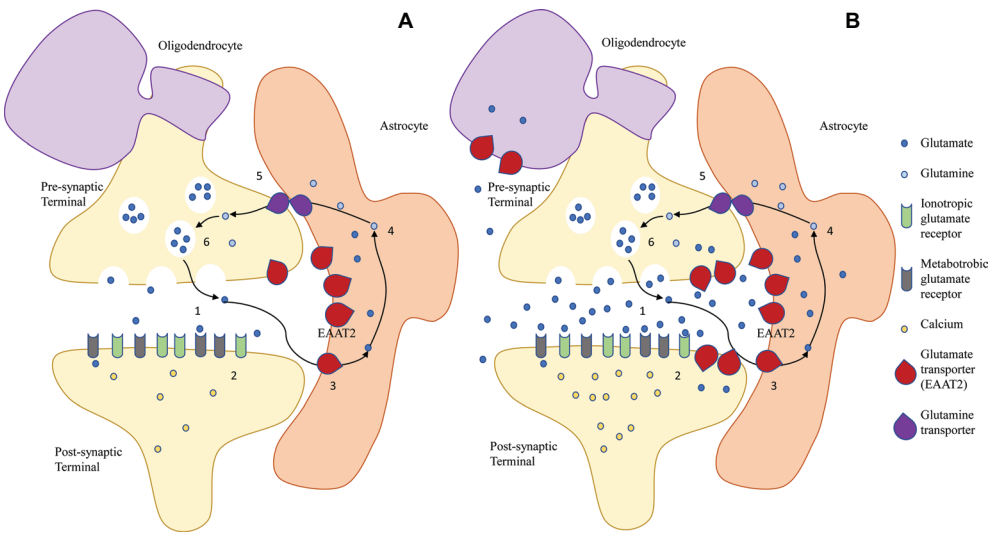

FIGURE 1 | The glutamate/glutamine cycle in **(A)** physiological conditions and **(B)** excitotoxic conditions in the immature brain. **(A)** In the mature healthy brain, glutamate is released by exocytosis from the pre-synaptic neuronal terminal into the synapse (1), and it binds to post-synaptic ionotropic (NMDA, AMPA, and kainate receptors) and metabotropic (mGluR) glutamate receptors, inducing Ca^{2+}-mediated signaling cascades that result in cellular responses (2). Extracellular glutamate is taken up primarily by astroglial EAAT2 (3) and converted to glutamine (4), which is shuttled back to the pre-synaptic terminal *via* glutamine transporters (5). Here, glutamine is converted back to glutamate (6). **(B)** During excitotoxicity, a combination of increased neuronal release and decreased astroglial uptake lead to a rise of extracellular glutamate levels, leading to overactivation of the post-synaptic glutamate receptors, Ca^{2+} overload, and activation of apoptotic pathways. Reversal of transport of astroglial transporters may also contribute to the accumulation of extracellular glutamate. In the immature brain, upregulation of the glutamate transporters in underdeveloped neurons and oligodendrocytes may contribute to their selective vulnerability.

periventricular white matter of newborn rodents, rabbits, and kittens produces patterns of perinatal brain injuries similar to those seen in humans (McDonald et al., 1988; Innocenti and Berbel, 1991a,b; Marret et al., 1995; Gressens et al., 1996; Acarin et al., 1999; Follett et al., 2000). On the other hand, pharmacological inhibition of glutamate receptors before or immediately after an hypoxic-ischemic insult is neuroprotective in both preterm (Follett et al., 2004; Manning et al., 2008) and term (Hagberg et al., 1994; Follett et al., 2000) brain injuries. Indeed, one of the mechanisms through which magnesium sulfate is thought to exert neuroprotection is by preventing excitotoxic damage through NMDA receptor blockade (Lingam and Robertson, 2018).

In vivo Evidence of Glutamate Excitotoxicity

Evidence of *in vivo* disturbance of glutamate signaling has been produced for animal models of hypoxic-ischemic brain injury. In a rat model of mild white matter injury near term, a rise in extracellular glutamate is observed in the acute phase after hypoxia-ischemia, with oligodendrocytes and axons representing the major sources of extracellular glutamate and astrocytes failing to take up excess glutamate (Back et al., 2007a). Similarly, repeated umbilical cord occlusion in the near-term fetal sheep causes periventricular white matter injury, the extent of which correlates with extracellular local glutamate levels (Loeliger et al., 2003). Notably, the largest increase in glutamate occurred over the hours after the insult, a delayed increase that suggested impaired glutamate transport. In a piglet model of hypoxic-ischemic encephalopathy at term, glutamate levels in the basal ganglia were shown to change in two phases: an early increase in the first 6 hours was followed by transient and slight recovery by 12 hours, possibly due to the self-protective glutamate transport mechanisms and conversion to glutamine in astrocytes; a further increase occurred after a day, possibly through cells bursting due to reperfusion injury and reversal of glutamate transport in the late stages of disease (Dang et al., 2017). In humans, elevated glutamate levels have been reported in the cerebrospinal fluid and basal ganglia of asphyxiated newborns (Riikonen et al., 1992; Hagberg et al., 1993). Moreover, elevated glutamine levels have been found in MRI-defined punctate necrotic white matter lesions (Wisnowski et al., 2013). Glutamate is taken up into astrocytes for conversion into glutamine and shuttling back to neurons. The finding of elevated glutamine rather than glutamate may be due at least in part to the temporal lag between insult and measurement. An important limitation of *in vivo* glutamate measurements in preterm newborns is that the peak window of glutamate changes is probably missed, because magnetic resonance measurements are likely to be carried out long after the initial insults in newborns that have already become sick. As such, these findings suggest that disrupted glutamate homeostasis persists in the subacute phase in moderate necrotic white matter injury. Although a relatively small subset of the newborns with punctate lesions also had evidence of cysts, no studies to date have measured glutamatergic metabolism specifically in newborns with severe cystic white matter injury.

Glutamate Excitotoxicity Following Hypoxia-Ischemia

Glutamate homeostasis can be disrupted by an acute hypoxic-ischemic event, and the phases of the subsequent excitotoxic injury are well described. During the primary energy failure, oxygen and blood deprivation lead to impairment of ATP production due to failure of oxidative phosphorylation. Astrocytes, with their unique oxidative capacity and ability to upregulate ATP production, are central to maintaining energy metabolism during the first stage of ischemia (Dienel and Hertz, 2005). Impairment of the ATP-dependent Na^+/K^+ pumps leads to loss of the electrochemical gradient across the cell membrane. If the insult is severe, some cells may die at this early stage *via* necrosis, due to influx of ions and water, cell swelling, and bursting. Within hours, the necrotic injury due to severe energy failure leads to death of all cellular elements and develops into the white matter cysts (Back, 2017). Depolarization of the cell membrane activates Ca^{2+} channels in the pre-synaptic terminal, triggering vesicular release of glutamate in the synapse. In astrocytes, hypoxia-ischemia leads to a failure in the astrocytic glutamate uptake system, which also relies on Na^+/K^+ gradients. The combination of increased synaptic release and reduced astrocytic uptake leads to accumulation of glutamate in the synaptic space and overactivation of post-synaptic ionotropic and metabotropic glutamate receptors (Volpe, 2008). The subsequent intracellular Ca^{2+} influx triggers activation of phospholipases, endonucleases, proteases, and nitric oxide synthase, with degradation of cellular and extracellular structures, and generation of harmful free radicals and reactive oxygen and nitrogen species. Glutamate leaking outside the synapse activates extrasynaptic NMDA receptors, which, contrarily to the pro-survival action of synaptic NMDA receptors, promotes excitotoxic cell death even further (Parsons and Raymond, 2014). This excitotoxic-oxidative cascade eventually leads to cell damage or death *via* necrosis, apoptosis, and autophagy in the secondary phase of injury (Olney, 1969; Benveniste et al., 1984; McDonald and Johnston, 1990; Choi, 1992; Thornton et al., 2012; Back, 2017; Descloux et al., 2018) (**Figure 1**).

Glutamate Excitotoxicity Following Inflammation

In preterm brain injury, comorbidities stimulating inflammation are thought to contribute to disruption of glutamate homeostasis and potentiation of excitotoxicity. TNFα, for example, is one of the most studied cytokines and is emerging as a key link between inflammation and glutamate excitotoxicity (Olmos and Llado, 2014). TNFα has both neuroprotective and neurotoxic effects depending on the different signaling pathways activated by the different receptors. In fact, pharmacological inhibition or genetic deletion after a combined inflammatory and excitotoxic insult is neuroprotective (Aden et al., 2010; Kendall et al., 2011), but knocking out TNFα receptors in the mouse increases susceptibility to hypoxic-ischemic injury (Bruce et al., 1996). TNFα potentiates glutamate excitotoxicity *in vitro via* complex and interacting mechanisms involving crosstalk between neurons and glial cells and leading to vicious cycles of glutamate and cytokine release.

In neurons, TNFα increases the excitatory strength at the synapse by increasing cell surface expression of glutamate receptors and their permeability to Ca^{2+}, while also decreasing expression of inhibitory $GABA_A$ receptors (Olmos and Llado, 2014). In microglia, TNFα stimulates autocrine release of TNFα and glutamate by upregulating glutaminase and from hemichannels of gap junctions (Takeuchi et al., 2006). In astrocytes, TNFα stimulates glutamate release *via* prostaglandin E2 and exacerbates impairment of glutamate transport (Bezzi et al., 1998). Cheung et al. (1998) suggested that glutamate concentration may be key in determining the pathways of cell death, with higher glutamate concentrations preferentially triggering necrosis and lower concentrations leading to apoptosis. Either way, even transient excess of glutamate can start a number of events that ultimately cause death or damage of vulnerable cell populations (Ottersen et al., 1996).

Glutamate Excitotoxicity and Perinatal Brain Injuries

The patterns of excitotoxic injury tend to be different in the preterm and term brain. Experimental evidence suggests that the main cellular target of excitotoxic injury in the preterm brain is pre-oligodendrocytes (Volpe et al., 2011). Glutamate is highly toxic to pre-oligodendrocytes in cell culture and leads to cell death *via* free radical attack (Oka et al., 1993). The white matter in the rat is much more vulnerable to hypoxia-ischemia at preterm-equivalent age, when pre-oligodendrocytes are predominant, than at term-equivalent age, when mature oligodendrocytes are the major form (Back et al., 2002; Craig et al., 2003; Dean et al., 2011). Indeed, the patterns of hypoxic-ischemic white matter injury seem to be determined primarily by the timing of appearance (Buser et al., 2010) and spatial distribution (Riddle et al., 2006) of pre-oligodendrocytes rather than severity of ischemia itself. Pre-oligodendrocytes are strikingly more vulnerable than immature neurons of the cortex and caudate nucleus in moderate global ischemia in the preterm fetal sheep (Dean et al., 2013; McClendon et al., 2014). Immature neurons are also vulnerable, as NMDA receptors are functionally upregulated, more permeable to calcium and less sensitive to magnesium block (Jantzie et al., 2015).

In physiological conditions, the abundance of glutamate receptors in the white matter is key during early neuronal development, contributing to rapid growth and myelination. However, their abundance also confers increased vulnerability in excitotoxic conditions (Kaindl et al., 2009). Indeed, the selective vulnerability of subplate neurons compared to cortical neurons observed in a preterm model of hypoxia-ischemia has been suggested to originate from an increase of glutamate receptors in these neurons associated with early maturation (McQuillen et al., 2003). Similarly, it has been suggested that selective vulnerability of the deep grey matter and sensorimotor cortex in term hypoxic-ischemic encephalopathy could be related to peaking NMDA receptor expression and proximity to developing glutamatergic circuits (Rocha-Ferreira and Hristova, 2016). As such, developmental expression of key glutamatergic genes in the grey and white matter may contribute to the different patterns of excitotoxic injury (Volpe, 2008).

Overall, the potential sources of extracellular glutamate in the white matter include pre-oligodendrocytes, astrocytes, neurons, ependymal cells, and cells of the choroid plexus (Back and Rosenberg, 2014). While therapies targeting excitotoxicity have so far mostly focused on glutamate receptor blockade, targeting glutamate transport is gathering interest as a potential avenue for neuroprotection by counteracting glutamate accumulation in the first place (Tilleux and Hermans, 2007; Kim et al., 2011; Fontana, 2015; Takahashi et al., 2015).

GLUTAMATE TRANSPORT: FOCUS ON EAAT2/GLT-1

Maintaining the baseline extracellular glutamate concentrations in the nanomolar range is essential to avoid extracellular glutamate build-up. The brain has no known enzymatic mechanism to metabolize glutamate in the extracellular space, and simple diffusion over short distances is thought to bring only a minor contribution. Hence, the brain relies substantially on intracellular glutamate uptake, and astrocytes provide by far the largest contribution to preventing excitotoxicity through expression of glutamate transporters (Danbolt, 2001; Tzingounis and Wadiche, 2007; Vandenberg and Ryan, 2013). Given their crucial role, it is not surprising that expression of astrocytic glutamate transporters is constitutively high (Zhou and Danbolt, 2013). Crosstalk between neurons and glia relies on tightly controlled extracellular glutamate homeostasis, and it is becoming increasingly evident that neuron-glia interactions are central to both the kinetics of glutamatergic synaptic activity in physiological (Fontana, 2015) and excitotoxic conditions (Carmignoto, 2000). Glutamate is released by astrocytes in immature rat optic nerve in ischemia *in vitro* (Wilke et al., 2004). Moreover, glutamate transport has been observed in immature axons (Arranz et al., 2008), and impairment has been reported in pre-oligodendrocytes during hypoxia-ischemia, providing a potential mechanism of excitotoxic vulnerability (Oka et al., 1993; Domercq et al., 1999; Fern and Moller, 2000; Deng et al., 2003; Desilva et al., 2007, 2009). The importance of glutamate transport to the integrity of oligodendrocytes and white matter is supported by evidence of extensive excitotoxic injury in oligodendrocytes and axons with experimental inhibition of glutamate transport in the optic nerve *in vivo* (Domercq et al., 2005).

The five members of the excitatory amino acid transporter (EAAT) family carry out most of the glutamate clearance in the central nervous system (Anderson and Swanson, 2000), especially EAAT1 (SLC1A3, rodent orthologue Glast) and EAAT2 (SLC1A2, rodent orthologue Glt-1) (Bristol and Rothstein, 1996). EAAT2 is the major glutamate transporter in the forebrain, except in the cerebellum, circumventricular organs, and retina, where EAAT1 is prevalent. In physiological conditions, both EAAT1 and EAAT2 are expressed predominantly by astrocytes and localized to the cellular membrane in the adult brain (Danbolt, 2001; Roberts et al., 2014; Takahashi et al., 2015). The high concentration (1 mg/g rat brain tissue), ubiquity

(1% of total CNS protein in the adult brain), and high degree of conservation across mammalian species are all indications of physiological importance of EAAT2/Glt-1 (Danbolt, 2001; Fontana, 2015; Danbolt et al., 2016). Unsurprisingly, it is expressed at high density near glutamatergic synapses in developing hippocampal astrocytes, with density and vicinity increasing with neuronal activity (Benediktsson et al., 2012). This transmembrane transporter carries out glutamate uptake through a high affinity energy-dependent process driven by Na^+ and K^+ gradients. Specifically, glutamate and aspartate are co-transported inside the brain cells with 3 Na^+ and 1 H^+ for the antiport of 1 K^+. EAAT2 is also a selective anion channel, transporting Cl^- anions during intermediate conformations, uncoupled from the flux of glutamate (Fontana, 2015).

Several lines of evidence support the central role of EAAT2 expression/function in maintaining extracellular glutamate homeostasis. Pharmacological inhibition of glutamate transport, including EAAT2, leads to rapid extracellular glutamate increase *in vitro* (Jabaudon et al., 1999) and extended post-synaptic activation mediated by NMDA receptors (Lozovaya et al., 1999). Genetic deletion of Glt-1 *via* constitutive knockout in the mouse leads to lower body weight, seizures, acute cortical injury in the forebrain, and increased mortality from the second/third postnatal week (Tanaka et al., 1997). Brain tissue from this mouse shows much lower (5%) glutamate transport activity than wild-type, suggesting that Glt-1 is responsible for up to 95% of glutamate transport. This is confirmed by the ability of Glt-1 antibodies to remove 90% of the transport activity in forebrain tissue (Haugeto et al., 1996). Other Glt-1 knockouts have confirmed the obvious phenotype, with lower life span, lower body and brain weight, mild loss of CA1 neurons in the hippocampus, and severe focal neuronal loss in layer II of the neocortex and focal gliosis (Kiryk et al., 2008). A conditional knockout mouse with selective deletion of Glt-1 reproduces this phenotype while ruling out developmental adaptations (Zhou et al., 2014). Heterozygote knockouts, on the other hand, show halved concentrations of Glt-1, but no apparent morphological brain changes, despite an increased risk of traumatic spinal cord injury (Kiryk et al., 2008; Lepore et al., 2011). Inhibition with antisense oligonucleotides *in vitro* and *in vivo* induces a rise in extracellular glutamate, excitotoxic injury, and progressive paralysis (Rothstein et al., 1996). On the other hand, selective overexpression in astrocytes is neuroprotective during ischemia (Chao et al., 2010).

Studies of EAAT2 expression point to different patterns depending on cell type, region, developmental age, species, and methodology used (DeSilva et al., 2012). In the adult rat, Glt-1 is expressed in the forebrain, especially in the hippocampus, cortex, striatum, and thalamus as well as in fibrous astrocytes in the white matter (Lehre et al., 1995). The transporter is expressed predominantly by astrocytes but also pre-synaptic axon terminals in the rodent hippocampus and somatosensory cortex (Danbolt, 2001; Chen et al., 2004; Furness et al., 2008; Melone et al., 2009; de Vivo et al., 2010; Danbolt et al., 2016). Neuronal EAAT2 represents no more than 10–20% total EAAT2 (Furness et al., 2008; Danbolt et al., 2016), and while being implicated in adult neuropsychiatric disorders (O'Donovan et al., 2017), neuronal knockout barely affects total Glt-1 protein levels and mouse development (Petr et al., 2015). Conversely, astrocytic knockout leads to a reduction of protein levels to a fifth in the forebrain, lower body weight and increased epilepsy and mortality.

Developmental Expression of EAAT2

The scenario may be at least in part different in the preterm brain, where transient but more prominent neuronal and pre-oligodendrial expression is observed. During development, dynamic and species-specific changes in both cellular and regional expression have been observed, suggesting that glutamate transporters may be both regulated by and involved in brain development (e.g., participation in the development of the topographic organization). As expected, these changes in rodent Glt-1 expression correspond to changes in total glutamate uptake activity (Ullensvang et al., 1997). Briefly, Glt-1 expression is low until after birth, except for a transient peak of expression in developing axons and oligodendrocytes around mid-late gestation. Glt-1 is expressed *in vivo* in rat pre-oligodendrocytes, whereas it is no longer detectable in mature oligodendrocytes (DeSilva et al., 2009). Transient neuronal expression is also seen around mid-late gestation in the mouse (Sutherland et al., 1996; Yamada et al., 1998), rat (Furuta et al., 1997), and sheep (Northington et al., 1998). In the fetal rat, Glt-1 is expressed in the amygdala and hippocampus, as well as white matter tracts interconnecting neocortex, basal ganglia, and thalamus (Furuta et al., 1997). In the fetal sheep, Glt-1 is found not only in white matter tracts but also in neuronal bodies and extended to the subplate, cranial nerve nuclei, basal ganglia, and cerebellar cortex, highlighting potential species differences in cellular expression during development (Furuta et al., 1997; Northington et al., 1998, 1999). In the newborn rat at P1, Glt-1 levels are the highest in the spinal cord and moderate in the hippocampus and hypothalamus. Expression increases dramatically from the second postnatal week throughout the central nervous system, especially in the cortex, striatum, caudate nucleus, and hippocampus, reaching adult levels by weeks 4–5 (Rothstein et al., 1994; Levy et al., 1995; Shibata et al., 1996; Sutherland et al., 1996; Furuta et al., 1997; Ullensvang et al., 1997). Astrocyte selectivity is established in the postnatal period in rodents and around mid-late gestation in sheep (Furuta et al., 1997; Takasaki et al., 2008). Nonetheless, Glt-1 is still detected in immature axons at P14–17 (Arranz et al., 2008). The significant developmental changes in Glt-1 after birth may explain why the Glt-1 knockout mice seem to develop normally for the first few weeks and develop seizures and brain injury during postnatal week 3, with many dying by week 4 (Tanaka et al., 1997; Takasaki et al., 2008).

A limited number of studies have investigated developmental regulation of EAAT2 in humans. DeSilva et al. (2012) found that, among EAAT1–3, expression of EAAT2 undergoes particularly prominent maturational changes in post-mortem cortex tissue of preterm and term newborns without neurological disease, all the way into childhood. Consistent with animal

studies, EAAT2 expression is generally low until birth and is limited to glia limitans, layer I-III fine astrocytes, and some neuron populations. EAAT2 was found not only in axons but also in the cell body and dendrites of certain neuron populations from as early as 23 gestational weeks up until term and, in some cases, until 8 postnatal months. These neuron populations are layer V pyramidal neurons, layer I neurons (putative Cajal-Retzius cells), and subplate neurons (DeSilva et al., 2012). A great proportion of these neuronal populations is glutamatergic, and it has been suggested that this transient neuronal EAAT2 expression is critical for establishing and orchestrating excitatory transmission during maturation and migration of cortical neurons. Similarly, it could also provide the basis for selective vulnerability to premature excitotoxic injury due to expression of glutamate transporters, which may reverse transport and become sources of extracellular glutamate (Takasaki et al., 2008; DeSilva et al., 2012), as discussed below. This is supported by evidence of selective vulnerability of layer V pyramidal neurons and subplate neurons in human and rat preterm white matter injury (McQuillen et al., 2003; Andiman et al., 2010). The same group reported EAAT2 expression in pre-oligodendrocytes in human fetal white matter at 32 weeks of gestation, during the peak time for premature brain injury, but not at 7 months old, consistent with rat studies (Desilva et al., 2007). EAAT2 expression appeared in the astrocytes of the developing cortex at 41 postconceptional weeks, increasing steeply in the first 1.5 years (DeSilva et al., 2012). Taken together, these findings suggest that the expression of EAAT2/Glt-1 undergoes substantial changes during development and that these changes may contribute to the selective vulnerability of cellular (e.g., immature oligodendrocytes and neurons) and regional (e.g., white matter tracts, hippocampus) targets in preterm brain injury.

EAAT2 and Preterm Brain Injury

Following severe energy failure, the dissipation of the transmembrane gradient impairs astrocytic EAAT2, which relies on transmembrane Na^+/K^+ gradients. This disruption may involve both quantity and quality of transport activity, i.e., it can manifest as decreased expression and/or impairment of glutamate transport function with establishment of reverse transport. Reverse transport has an outward direction and is driven by the transmembrane gradient of excitatory amino acids independently of ATP and Ca^{2+} (Nicholls and Attwell, 1990; Szatkowski et al., 1990; Levi and Raiteri, 1993). In this scenario, glutamate transporters become themselves a major source of extracellular glutamate, potentially turning into key contributors of excitotoxic injury in any cells expressing them (Domingues et al., 2010) (**Figure 1**). While its significance to preterm brain injuries remains to be explored, the importance of reverse transport is supported by evidence that ischemic cell death in the rat striatum can be blocked by an inhibitor of reverse Glt-1 transport (Seki et al., 1999). Moreover, knockout mice lacking Glt-1 are more vulnerable to neuronal death after a short, severe episode of ischemia than wild-type mice, suggesting that Glt-1 is essential for neuroprotection when ischemia is acute; on the other hand, wild-type mice expressing Glt-1 are more vulnerable to neuronal death than mice lacking Glt-1 during extended, chronic ischemia, suggesting that Glt-1 (*via* reverse transport) becomes neurotoxic when ischemia is prolonged (Mitani and Tanaka, 2003).

Consistent with impairment of glutamate transport, a decrease in glutamate uptake is seen in the hippocampus of rat pups exposed to intrauterine hypoxia following caesarean delivery (Frizzo et al., 2010) and in the cortex, basal ganglia and thalamus of newborn piglets exposed to hypoxia (Jantzie et al., 2010). Loss of Glt-1 expression and/or function has been reported in astrocyte cultures during hypoxia (Dallas et al., 2007) as well as in the adult rat cortex and hippocampus after ischemia (Torp et al., 1995; Rao et al., 2001a,b). In a small study of term-equivalent rats, astrocytic Glt-1 was suppressed in the initial 12 hours in the ischemic core of both the hippocampus and the neocortex, recovered after 48 hours only in the hippocampus, followed by astrogliosis at 72 hours (Fukamachi et al., 2001). In a piglet model of hypoxic-ischemic encephalopathy at term, canonical suppression of Glt-1 in astrocytes of the striatum and hippocampus was accompanied by upregulation in neurons of the striatum (Martin et al., 1997b; Danbolt, 2001; Pow et al., 2004; Desilva et al., 2007, 2012). The striatum is known to be selectively vulnerable to excitotoxicity at term, and this may suggest a potential neuronal response to locally increasing extracellular glutamate levels (Martin et al., 1997a). In P6 rats, exposure to hypoxic preconditioning led to upregulation of Glt-1 in the cortex and suppression in the striatum, with no detectable changes in the hippocampus (Cimarosti et al., 2005). Glt-1 was also suppressed in the white matter in a preterm mouse model of chronic hypoxia, although this model was not subjected to ischemia and showed no sign of reactive astrogliosis (Raymond et al., 2011). Moreover, hypoxia has been found to alter the expression of Glt-1 splice variants in mouse brain and neurons of newborn pigs (Munch et al., 2003; Pow et al., 2004).

Exposure of mouse astrocytes, rat microglia, and human blood macrophages to the bacterial endotoxin lipopolysaccharide (LPS) and the pro-inflammatory cytokine TNFα has been found to enhance EAAT2 expression and glutamate uptake function *in vitro* (Rimaniol et al., 2000; Persson et al., 2005; O'Shea et al., 2006). On the other hand, TNFα suppresses both glutamate uptake and EAAT2 in a dose-dependent manner (via NF-κB) in human fetal astrocytes (Fine et al., 1996; Liao and Chen, 2001; Su et al., 2003). TNFα also selectively suppresses EAAT2 *via* NF-κB during hypoxia *in vitro* (Boycott et al., 2008).

An important finding is that EAAT2 is upregulated in the reactive astrocytes and macrophages of post-mortem human brain tissue from preterm babies with white matter injury compared to controls, suggesting a possible response to hypoxia-ischemia and/or inflammation in the preterm brain (Desilva et al., 2008). Pre-oligodendrocytes in both cases and controls expressed EAAT2, with no qualitative differences in expression, although function was not measured. Upregulation of EAAT2 in reactive astrocytes and macrophages in preterm white matter injury may be an adaptive mechanism to counteract excitotoxicity, or it could be a secondary mechanism due to gliosis. Whether in chronic white matter injury, this upregulation contributed to excitotoxicity *via* transport reversal remains to

be established. Further studies are needed to elucidate how perinatal hypoxia-ischemia and infection/inflammation affect EAAT2 homeostasis, separately and in combination. Interestingly, genome-wide gene expression analysis of reactive astrocytes in two adult mouse models of ischemic stroke and LPS-induced neuroinflammation revealed that at least half of the altered gene expression is specific on the insult, with indication that reactive astrocytes may be neuroprotective in ischemia but detrimental in neuroinflammation (Zamanian et al., 2012). Overall, candidacy of EAAT2 is supported by the fact that dysregulation is implicated in several neurological, neurodegenerative, and psychiatric disorders thought to involve glutamate excitotoxicity (i.e., transient cerebral ischemia, ischemic stroke, epilepsy, traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, chronic pain, Huntington's disease, HIV-associated cognitive disorder, glioma, major depression, schizophrenia, and addiction) (Danbolt, 2001; Beart and O'Shea, 2007; Fontana, 2015; Karki et al., 2015; Takahashi et al., 2015; Verkhratsky et al., 2016; Zhang et al., 2016; Zhou et al., 2016; Goodwani et al., 2017; O'Donovan et al., 2017; Assefa et al., 2018; Fogarty, 2018; Kim et al., 2018; Parkin et al., 2018).

A better understanding of the role of glutamate transport in preterm brain injuries will require further investigations of EAAT1 in the cerebellum. EAAT1 is highly expressed in cerebellar astrocytes, particularly Bergmann's glia (Lehre et al., 1995; Danbolt, 2001). The processes of these cells ensheath the Purkinje cell synapses, which have been suggested to be selectively vulnerable to excitotoxicity induced by hypoxia-ischemia (Harding et al., 1984; Shibata et al., 1996). Indeed, EAAT1 is developmentally upregulated from 23 weeks gestation, possibly in conjunction with the maturation of the Purkinje cells. Importantly, EAAT1 undergoes rapid changes in hypoxic-ischemic encephalopathy at term, with a decrease in the molecular layer and an increase in the Purkinje and inner granule cell layer at an early stage. This increase becomes marked at a later stage, potentially pointing to an adaptive neuroprotective mechanism against excitotoxicity (Inage et al., 1998).

Mechanisms leading to loss of expression and/or function are likely to be complex. Ying's (1997) "deleterious network hypothesis" (1997) suggests that glutamate build-up may lead to detrimental vicious cycles. For example, receptor overactivation may lead to increased energy consumption and oxidative damage, which is known to impair glutamate transporters' activity and expression, potentially leading to reverse transport with further glutamate release. Ion flux may cause cell swelling, leading to impaired energy metabolism (Danbolt, 2001). Inflammation may further potentiate the risks of excitotoxicity *via* glutamate transport suppression, including selective effects on EAAT2 (Aden et al., 2010; Kapitanovic Vidak et al., 2012). Evidence to date supports the concept of suicide loops in pre-oligodendrocytes, which could provide both the source and the target for excitotoxic injury in the preterm brain. In this context, the combination of developmental upregulation of EAAT2 and establishment of reverse transport in the context of an energy failure could increase vulnerability of pre-oligodendrocytes to excitotoxic death (Back and Rosenberg, 2014). Similarly, transient expression in neuronal populations could feed into suicide loops and explain the loss of layer V pyramidal neurons accompanying necrotic PVL (Andiman et al., 2010). This is a different mechanism to that hypothesized in the mature brain, where the sources of glutamate killing neurons are thought to be other cells, including astrocytes and excitatory terminals (Lipton and Rosenberg, 1994) or, alternatively, retrograde degeneration from axonal injury. Astrocytes may have a delayed response due to their unique ability to use glycogen as a metabolic fuel during the initial stages of energy deprivation. In this scenario, extracellular glutamate concentrations may rise significantly only after depletion of glycogen stores in astrocytes (Grewer et al., 2008), with a subsequent steep rise in extracellular glutamate and excitotoxic cell death (Gouix et al., 2009). In chronic white matter injury, upregulation of astrocytic EAAT2 may be detrimental when accompanied by establishment of reverse transport. Experimental data are needed to evaluate these hypotheses.

POTENTIAL FUTURE DEVELOPMENTS

In summary, it is plausible that both up- and downregulation of EAAT2 contribute to disease, depending on animal model, developmental stage, type and severity of the insult, and comorbidities. Regulation and dysregulation of EAAT2 may occur at the level of transcription (including epigenetic regulation), translation, trafficking, transport, and degradation (Karki et al., 2015; Takahashi et al., 2015). Accordingly, treatments aiming at restoring EAAT2 expression are a current area of research in neuroprotection, alongside enhancement of the transport function (Fontana, 2015). Ceftriaxone, a licensed β-lactam antibiotic safe and tolerable for humans, enhances EAAT2 expression and has been shown to be neuroprotective in animal models of several adult excitotoxic disorders. Although no significant effects have been seen in clinical trials for amyotrophic lateral sclerosis and adult stroke, it is already widely used for the treatment of CNS infections in newborns and would therefore be a feasible drug to explore in the context of preterm neuroprotection. Guanosine enhances EAAT2 transport function and has shown neuroprotective effects in rat models of hypoxic-ischemic encephalopathy (Moretto et al., 2005, 2009) and adult cortical focal ischemia, *via* multiple mechanisms including prevention of free radical attack and pro-inflammatory response (Hansel et al., 2014, 2015). Several other expression and function enhancers of EAAT2 are currently gathering attention as a potential therapeutic approach for a variety of adult disorders and await exploration in the context of the newborn brain (Fontana, 2015). It is currently unknown whether EAAT2 enhancers would restore glutamate uptake or exacerbate reverse transport in the preterm brain. Combination therapies targeting different mechanisms and therapeutic windows will also need exploring, including more established (i.e., magnesium sulfate) and more exploratory therapies (e.g., anti-inflammatory treatment) (Ofek-Shlomai and Berger, 2014).

Genetic risk stratification and pharmacogenomic approaches focusing on interindividual differences in treatment response

are gathering interest and, as our healthcare systems develop, the integration of genomic data in clinical care seems an increasingly achievable goal (Rehm, 2017). Exploratory studies have implicated several functional genetic variants involved in glutamate excitotoxicity and inflammation in neurodevelopmental impairment, including as a sequelae of perinatal brain injuries (O'Callaghan et al., 2009, 2012, 2013; Wu et al., 2011; Kapitanovic Vidak et al., 2012). Among these, common genetic variants altering EAAT2 expression have been reported in association with cerebral palsy and neurodevelopmental delay in very preterm newborns (Rajatileka et al., 2017). Replication in larger samples, genome-wide designs and comparison with term brain injuries are needed to consolidate and expand the finding. Identification of panels of genetic variants that collectively increase risk of injury may be integrated with other types of clinical information and help identify high-risk pregnancies. Moreover, integration of genetic information has the potential to contribute to a more personalized approach to the care of the preterm newborn, with recent studies focusing on the interactions between genetic variants and responsiveness to antenatal magnesium sulfate therapy (Costantine et al., 2012; Clark et al., 2018). EAAT2 variants remain to be evaluated in this context.

Future *in vivo* studies will need to explore whether dysregulation of the main glutamate transporter, EAAT2, is central to the pathogenesis of preterm brain injuries or if it is a secondary process and whether the different cellular effects represent destructive or compensatory mechanisms. As explained by Danbolt (2001), "as long as one variable is not extreme, it will be the combination of several factors that will determine whether the ship will sink," and several different primary events/changes may share a final common pathway. Well-designed animal model studies will be needed to provide mechanistic evidence. Human post-mortem studies can provide insights into patterns of dysregulation of expression, function, and localization specific to the different types of perinatal brain injuries, though limited by confounding factors, post-mortem artifacts, reproducibility, and sample size. Promising preliminary findings on the neuroprotective effects of EAAT2 suggest that this is certainly an avenue worth exploring.

AUTHOR CONTRIBUTIONS

KL and SP contributed to the conception and design of the review. SP wrote the first draft of the manuscript. All authors revised, read, and approved the submitted version of the manuscript.

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